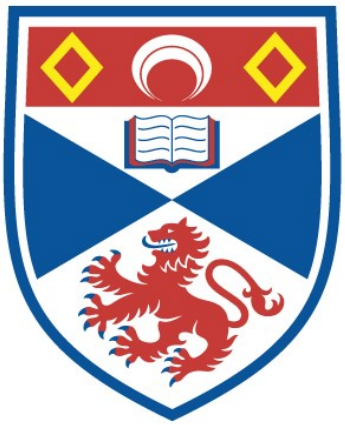
SOME STUDIES OF THE CHEMICAL REACTIVITY OF β-LACTAM ANTIBIOTICS

Derek I. Robinson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1981

SOME STUDIES OF THE CHEMICAL REACTIVITY OF β-LACTAM ANTIBIOTICS

A Thesis

presented for the degree of

DOCTOR OF PHILOSOPHY

in the

Faculty of Science

of the

UNIVERSITY OF ST ANDREWS

by

Derek I. Robinson

St Andrews

September 1981

ABSTRACT

Rate coefficients for the inactivation of several penicillins in acids and alkalis at 30°C have been determined by means of ultraviolet spectroscopy. The case of benzylpenicillin, though well investigated in the past, has been studied again and, in the light of new experiments, a scheme detailing the proportions of the various degradation products has been proposed. A number of substituted phenylpenicillins have been synthesised, and their reactions with acid have been investigated. Product analysis was carried out by means of TLC and NMR studies, the degradation products (penicilloic, penilloic, penillic and penicillenic acids) having been previously prepared and characterised. From the manner in which the phenyl substituent on the penicillin affects the rate coefficient, it is deduced that the mechanism of inactivation by acids involves a rate-determining intramolecular attack by the side-chain carbonyl group on the protonated β-lactam function. An intermediate oxazolone-thiazolidine structure is formed, which then reacts further by three different pathways. The proportions of the products obtained from the phenylpenicillins are different from those obtained from benzylpenicillin, principally because the phenylpenicillenic acids are easier to form and are less reactive towards acid than is benzylpenicillenic acid.

The imidazole-catalysed isomerisation of benzylpenicillin into penicillenic acid has been studied. This reaction was investigated using a number of substituted imidazoles and, from the rate equations which were obtained, a unified reaction mechanism has been proposed. This involves a rate-determining proton-transfer to an intermediate penicilloylimidazole complex, which is followed by fast ring opening

and expulsion of imidazole.

Some studies on the novel mono-cyclic β-lactam antibiotic nocardicin A have been carried out; its reaction in dilute and moderately-concentrated acid has been studied. It is proposed that the mechanism of hydrolysis involves a fast β-lactam cleavage followed by slower hydrolysis of the oxime function. Unlike most oximes, that of nocardicin A was found to hydrolyse faster as the acidity was increased. It is suggested that this unusual behaviour arises from the proximity of an amide group to the oxime. Similar behaviour has been observed for benzoyl formamide oxime.

DECLARATION

I declare that this thesis is based on the results of experiments carried out by me, that it is my own composition, and that it has not previously been presented for a Higher Degree.

This thesis describes the results of research carried out in the Department of Chemistry of the University of St Andrews and in the Laboratories of May and Baker Ltd of Dagenham, Essex , under the supervision of Dr A.R. Butler and Dr D.E. Wright, between October 1978 and August 1981.

Derek I. Robinson

CERTIFICATE

I hereby certify that Derek I. Robinson has spent twelve terms of research working under my supervision, has fulfilled the conditions of Ordinance General No. 12 and the Resolution of the University Court 1967, No. 1, and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Director of Research.

ACKNOWLEDGEMENTS

I wish to express my thanks to the following people:

To my supervisors Dr A.R. Butler and Dr. D.E. Wright for their help and encouragement throughout this project.

To the May and Baker Company for their hospitality to me over the past three years. In particular I should like to thank Dr P. Knowles, the staff of the Microanalysis Section and of the Pressure Reactions Section.

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Finally, I gratefully acknowledge financial assistance from the Science Research Council, who have provided the funds for the project.

CONTENTS

CHAPTER ONE

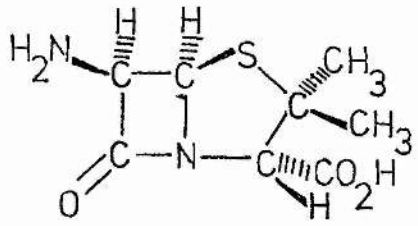
INTRODUCTION

A BRIEF HISTORICAL SURVEY

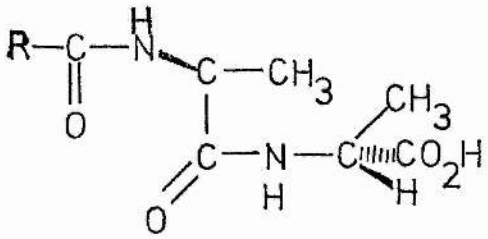
There can be little doubt that the discovery of penicillin by Sir Alexander Fleming in 1928[1] ranks as one of the most important developments of twentieth-century science. The discovery itself was a fortuitous one: some stray mould spores happened to enter Fleming's laboratory and contaminate his culture growths, at just the right temperature for them to produce their penicillin and thus cause the cell-lysis which Fleming observed. Nonetheless, this marks the origin of chemotherapy as we know it today. Since that time, thousands of strains of micro-organisms have been screened in the search for new and more powerful antibiotics.

The penicillins were among the first genuinely antibacterial chemicals to be used in medicine. Previous to their discovery, treatment of disease was largely the preserve of the vaccination techniques developed by Pasteur, where the body is stimulated to provide its own defence against infection. With the exception of the sulphonamide drugs, any chemical preparations which were used before 1940 were mainly of an antiseptic or disinfectant nature. Penicillin was the first relatively simple chemical (as distinct from complex biological products) to have a toxic effect on bacteria while being almost harmless to higher forms of life.

Ironically, Fleming's discovery lay dormant for about ten years, until its full significance was realised by Florey, Chain and their co-workers at the Sir William Dunn School of Pathology in Oxford. Their initial investigations established the enormous potential of the discovery,



(1)



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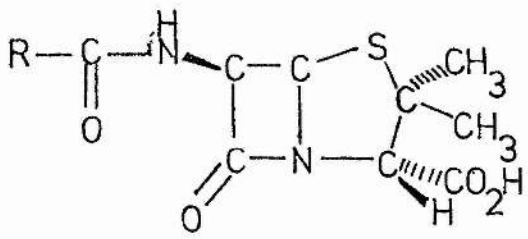
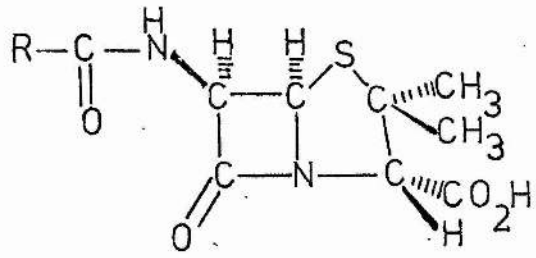


(3)

the importance of which was considerably augmented by the outbreak of the second world war. The research was greatly stepped up, and was soon joined by workers from the United States. The extent of this effort was such that, by the end of the war, considerable quantities of penicillin had been dispatched to the front for the relief of the wounded[3]. Also, the structure of the antibiotic had been fairly well established as the now-familiar fused β-lactam/thiazolidine ring system (3). This structure was considered very unusual at the time, and was greeted at first with some scepticism. It was, however, unequivocally confirmed by X-ray crystallographic studies[5]. The first total synthesis of a penicillin was achieved in 1957[6], but the overall yield was very low. (Since penicillin is available in high yield by fermentation processes, syntheses have never been of any commercial importance.)

It is the β-lactam structure which provides the key to the compound's remarkable antibacterial activity. Bacteria, in order to survive in hostile surroundings, build for themselves very tough cell walls; and they have evolved a special set of enzymes for this purpose. It is thought that the penicillin molecule reacts irreversibly with one of these enzymes, a transamidase, and thus terminates the cell-wall-building process[7]. Without the thick wall, osmotic pressure causes the cell to burst open: thus the bacteria are destroyed. Mammals have no counterpart to this cell-wall construction, therefore to them penicillin is non-toxic.

In reacting with the transamidase enzyme, the penicillin is thought to mimic an alanylalanine residue (2) - the enzyme's usual substrate. There is a structural and conformational similarity between the two molecules. The purpose of the enzyme is to break the C - N bond of the peptide. Analogously, when it meets the penicillin, it



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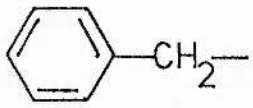
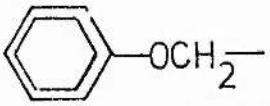
PENICILLINS

NATURAL

a) R = 

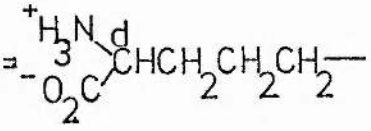
benzylpenicillin
penicillin G

b) R = 

phenoxymethylpenicillin
penicillin V

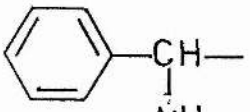
c) R = $CH_3CH{=}CHCH_2CH_2-$

2-pentenylpenicillin
penicillin F

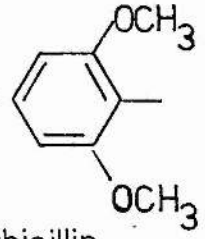
d) R = 

penicillin N

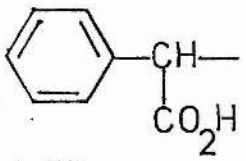
SEMISYNTHETIC

e) R = 

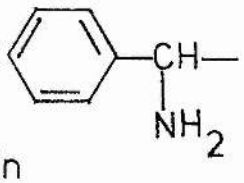
ampicillin

f) R = 

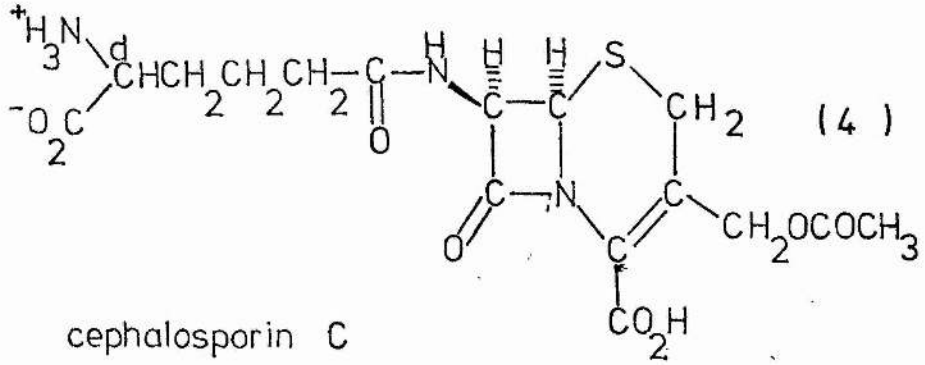
methicillin

g) R = 

carbenicillin

h) R = 

amoxycillin



(4)

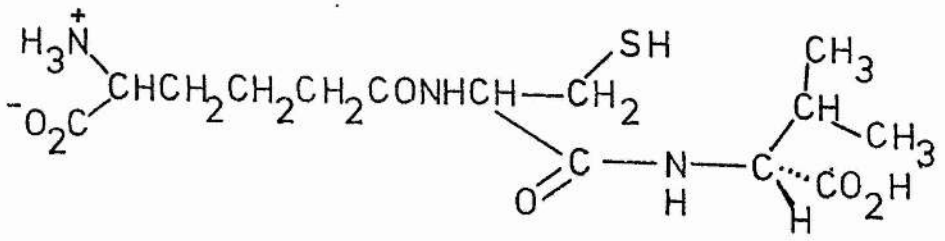
cephalosporin C

cleaves the β-lactam ring.

Several varieties of penicillin were found to be produced naturally by micro-organisms (eg 3a-d); but the range available was considerably widened after the isolation, in 1959, of the fundamental penicillin nucleus - now known as 6-aminopenicillanic acid (1)[8, 9]. Acylation of this compound with a variety of activated carboxylic acids led to a whole new series of 'semi-synthetic' penicillins (eg 3e-h). Many of these possess considerable advantages over the 'natural' products. For example, ampicillin (3e) is much more stable towards acids than is penicillin G (3a)[10, 11]. And methicillin (3f) possesses considerable resistance towards penicillinase[12, 13]. (This is an enzyme which is secreted by many bacteria and which cleaves the β-lactam ring of the penicillin, thus rendering it harmless to the bacteria[14, 15]. It is the cause of the resistance which bacterial species have developed towards penicillin.)

Penicillins usually display their greatest activity against gram-positive bacteria. However, in 1959, the first member of a new class of antibiotics, the cephalosporins, was isolated[16]. The structure of cephalosporin C (4) was determined in 1967[17, 18]. It is structurally similar to penicillin, having a fused β-lactam/dihydrothiazine ring system. Evidence suggests that both these antibiotics are produced by very similar biosynthetic pathways, and indeed have a common precursor in δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (5)[19]. Cephalosporins possess a much wider spectrum of antibiotic activity than do penicillins[20]. Total synthesis of a cephalosporin was first accomplished in 1966[21].

Unfortunately, the quantities of natural cephalosporin obtained from micro-organisms by fermentation are considerably



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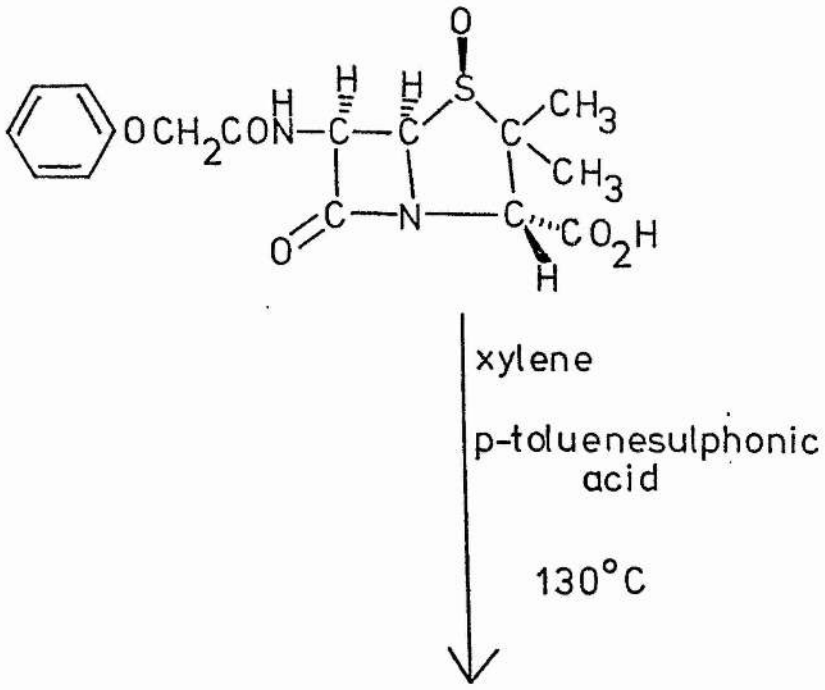
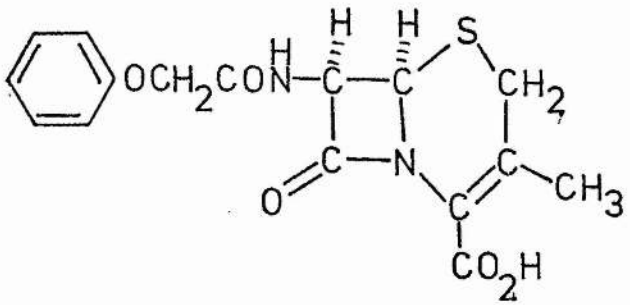
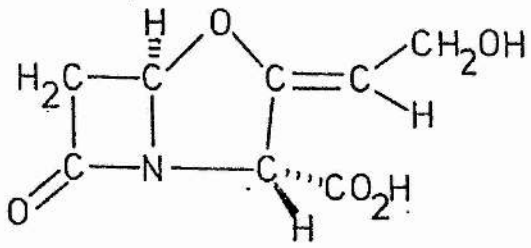




SCHEME 1



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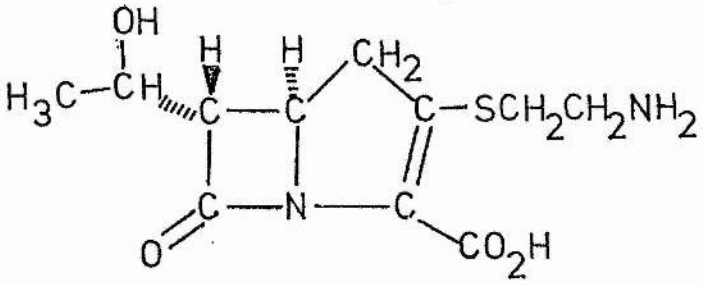

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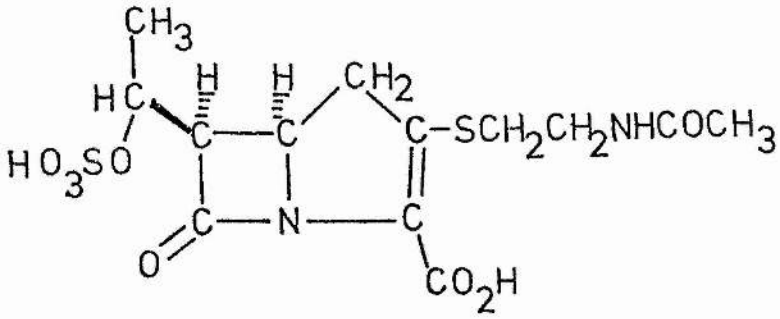

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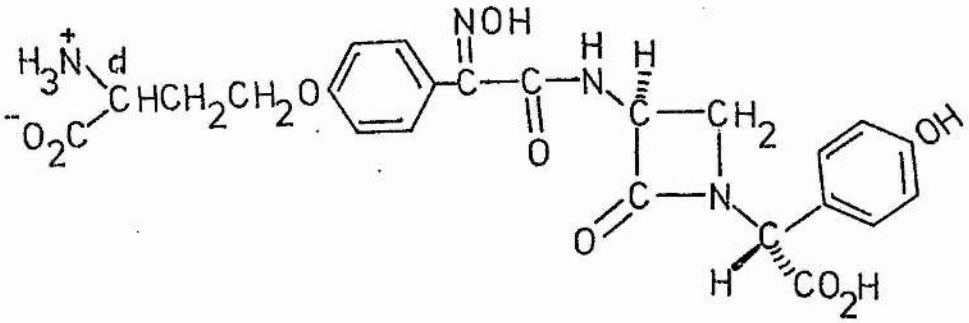


(9)

lower than those achieved for the penicillins. The latter are produced very efficiently, and cheaply, from a high-yielding strain of Penicillium chrysogenum. No cephalosporium strain so far isolated is nearly so efficient.

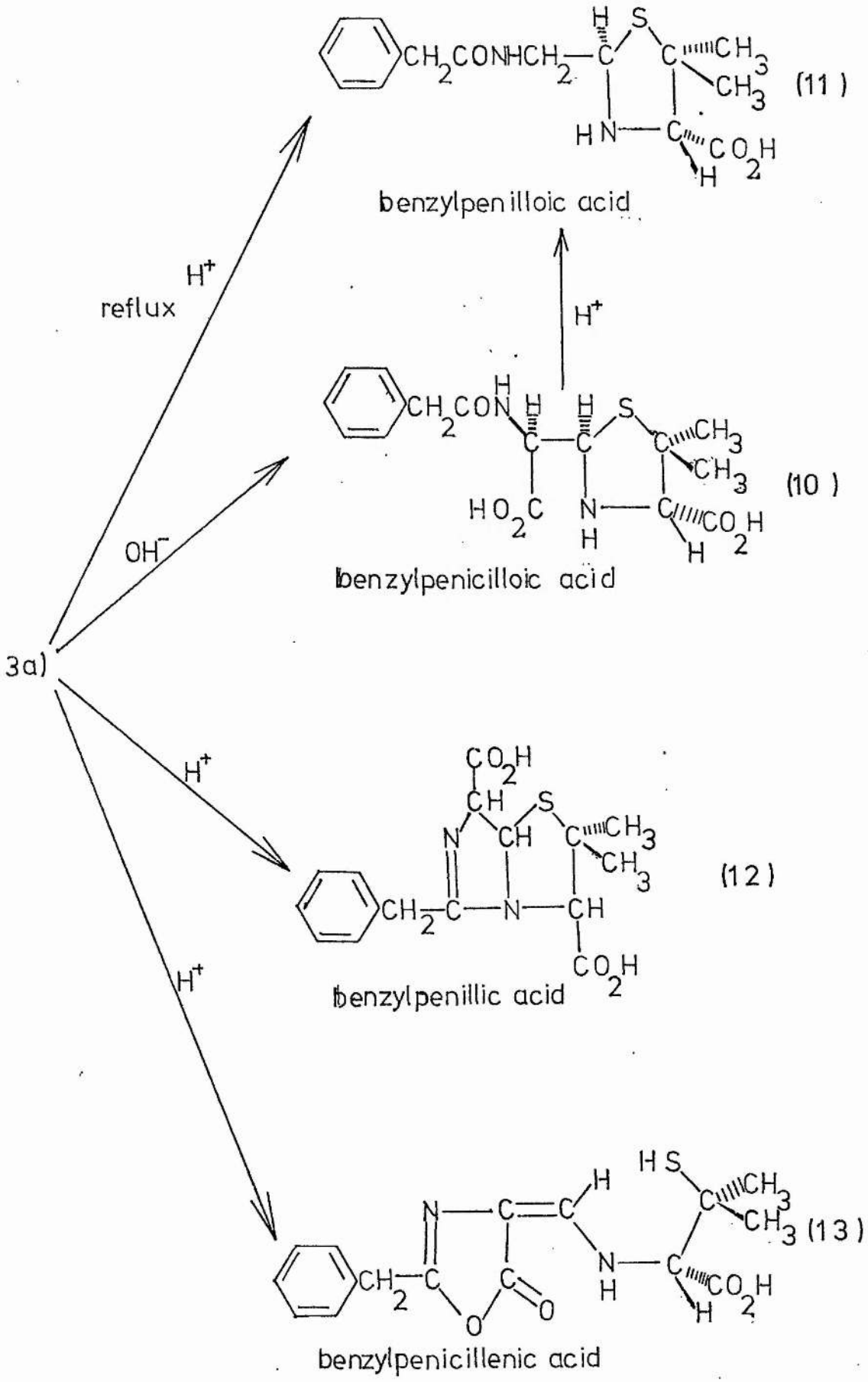
In 1963, however, it was discovered that cephalosporins could be formed by ring-expansion of penicillin sulphoxides (scheme 1)[22]. This reaction remains a key step in cephalosporin manufacture today.

The past twenty years have seen a continuing and widening search for new compounds containing the β-lactam moiety. Many have been found to be clinically useful, and indeed to possess superior qualities to the penicillins. Examples are clavulanic acid (6), a powerful inhibitor of β-lactamase[23, 24], thienamycin[25] (7), and olivanic acid (8)[26]. Even monocyclic β-lactams, such as nocardicin A (9), have been found to possess antibacterial properties[27]. Nevertheless, despite these new advances, penicillins remain, for all their deficiencies, among the most widely administered antibiotics, because they are much the cheapest to produce[28].

CHEMICAL REACTIONS OF THE PENICILLINS

The purpose of the present study has been to discover more concerning the chemical changes which penicillins undergo in aqueous media - particularly in acidic and basic solutions. It is concerned with the products formed in these solutions, and the rates at which they are formed.

A considerable store of knowledge has been accumulated over the years on this subject. Indeed, degradation studies were employed


benzylpenilloic acid
(11)
reflux
H+
H+
OH−
benzylpenicilloic acid
(10)
3a)
H+
benzylpenillic acid
(12)
H+
benzylpenicillenic acid
(13)

from the very beginning in structure determination, and the identification of reaction products gave valuable clues to the structure of the penicillins themselves[29]. Not only that, but some degradation products have important biological effects, and are thus compounds of interest in their own right.

Inevitably, most of the detailed degradation work has been concentrated on the reaction of penicillin G (3a), the most readily-available compound. This present work began with a reassessment of penicillin G and has proceeded to extend the ideas developed there to the phenylpenicillins (3, R = Ar). These, although they are mostly useless biologically, do help to provide valuable insights into the chemistry of penicillins as a whole.

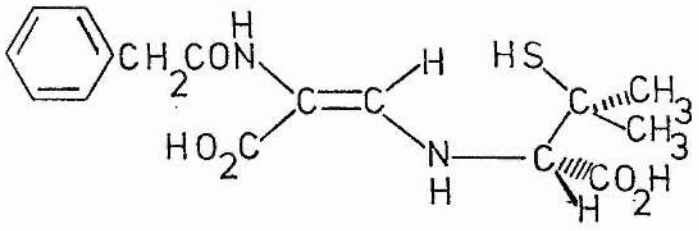
What follows now is a summary of the products which are known to arise from reactions of penicillin G.

In alkaline solutions the chemistry is very simple. The β-lactam function undergoes hydrolytic cleavage, giving one product only: known as benzylpenicilloic acid (10)[30]. This is also the product obtained when penicillin is inactivated by penicillinase[30]. It has no antibacterial properties.

The penicilloic acid is quite stable itself in alkaline solution, but in acidic solution it loses a molecule of carbon dioxide, to form benzylpenilloic acid (11)[30]. This compound can also be formed by refluxing benzylpenicillin in acid at pH 2[30].

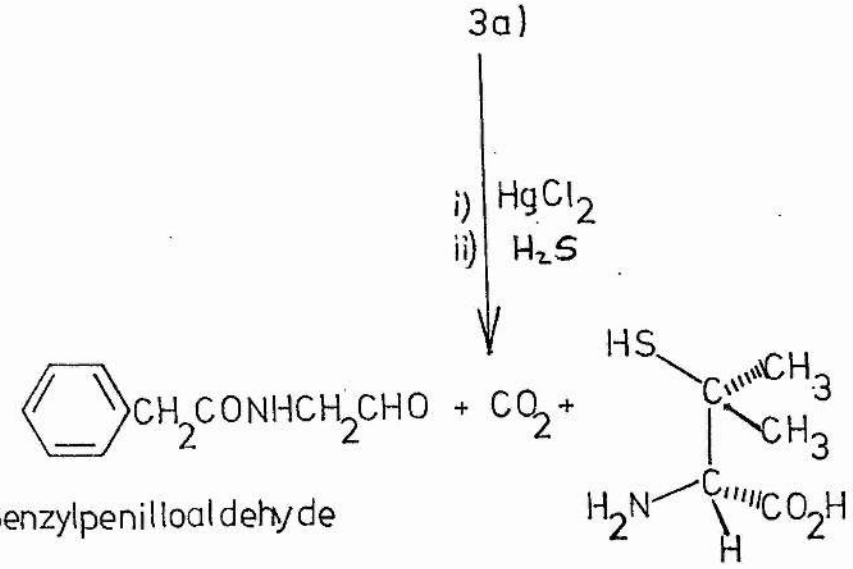
The action of acid on penicillin is, however, considerably more complicated. As well as penicilloic acid, there are several other products.

Of these, the most easily isolated is benzylpenillic acid (12). This is an isomer of penicillin which precipitates from a solution



(14)

benzylpenamaldic acid



benzylpenilloaldehyde

(16)

penicillamine

(15)

of the penicillin on standing at about pH 2[31]. There is evidence that, on standing for an even longer time in acid, this compound also decarboxylates to penilloic acid[32].

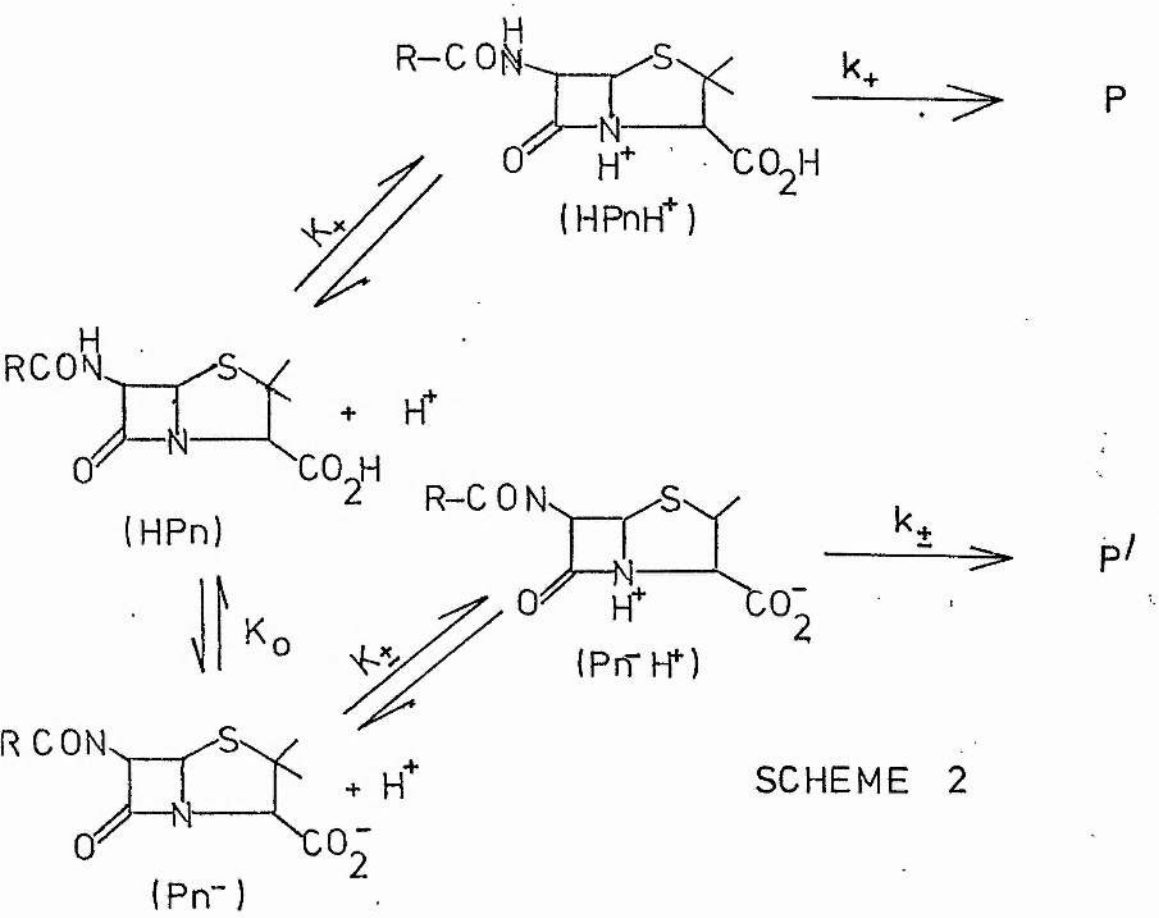
Another isomer is benzylpenicillenic acid (13). This was first isolated during an attempt at the total synthesis of penicillin G, and it was subsequently detected among the reaction products of the latter in acid[33]. One remarkable feature of this compound is an intense ultraviolet absorption band at 320 nm: a result of the compound's highly-conjugated structure.

Benzylpenicillenic acid has attracted a great deal of attention in its own right, since it is suspected to be the main cause of penicillin allergies[34]. It is found to be very unstable itself in both acidic and basic solutions (indeed far more so than the benzylpenicillin from which it is formed); and, depending on the pH, it can react to form penillic acid, penicilloic acid or penamaldic acid (14)[35]. Its stability in aqueous solution can be considerably enhanced by complexing with mercury ion[36], and in fact the compound can be isolated by reaction of the penicillin with mercuric chloride[34].

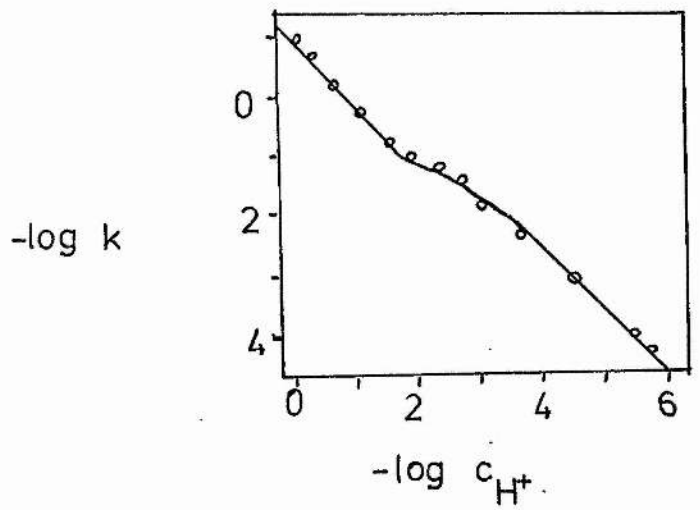
Mercuric chloride also has the effect of cleaving the penicillin molecule in two: the fragments being penicillamine (15) and a penilloaldehyde (16)[30].

KINETICS AND MECHANISM

There are numerous methods of determining the amount of penicillin present in any given sample, and thus of following the course of a reaction[37]. To obtain a direct measure of penicillin



SCHEME 2



pH - rate profile for inactivation of penicillin G 30° C 0·5M NaCl

concentration, a microbiological assay procedure is normally employed. Other methods work by determining the concentration of one or more of the reaction products. Among these latter are such diverse techniques as iodometric titration, acid-base titration, ultraviolet spectroscopy and polarography.

The first kinetic study of the inactivation of benzylpenicillin by acids seems to have been undertaken by Benedict et al.[38], who demonstrated that, at a fixed pH, this inactivation is first-order with respect to penicillin. Subsequently, Brodersen carried out a thorough study of this process, estimating penicillin by means of a turbidimetric assay[39, 40].

Brodersen postulated that the inactivation reaction proceeds via a protonation on the nitrogen of the β-lactam ring. Acidic solutions thus contain two species (which he denoted $HPnH^+$ and $Pn^{\pm}H^+$) which would spontaneously degrade (in some unspecified way) into biologically inactive substances (scheme 2). On the basis of this assumption he deduced that the pseudo-first-order rate constant k would be given by the following equation:

$$k = \left(\frac{k_+}{K_+} c_{H^+} + \frac{k_\pm}{K_\pm} K_o \right) \frac{c_{H^+}}{K_o + c_{H^+}}$$

where c_{H^+} is the hydrogen ion concentration

K_o is the dissociation constant of benzylpenicillin (with a value of $10^{-2.8}$ mol l^{-1})

k_+ is the first-order- rate constant for degradation of $HPnH^+$

$k_\pm$ is the first-order rate constant for degradation of $Pn^{\pm}H^+$

K_+ is the dissociation constant of $HPnH^+$

$K_\pm$ is the dissociation constant of Pn^-H^+

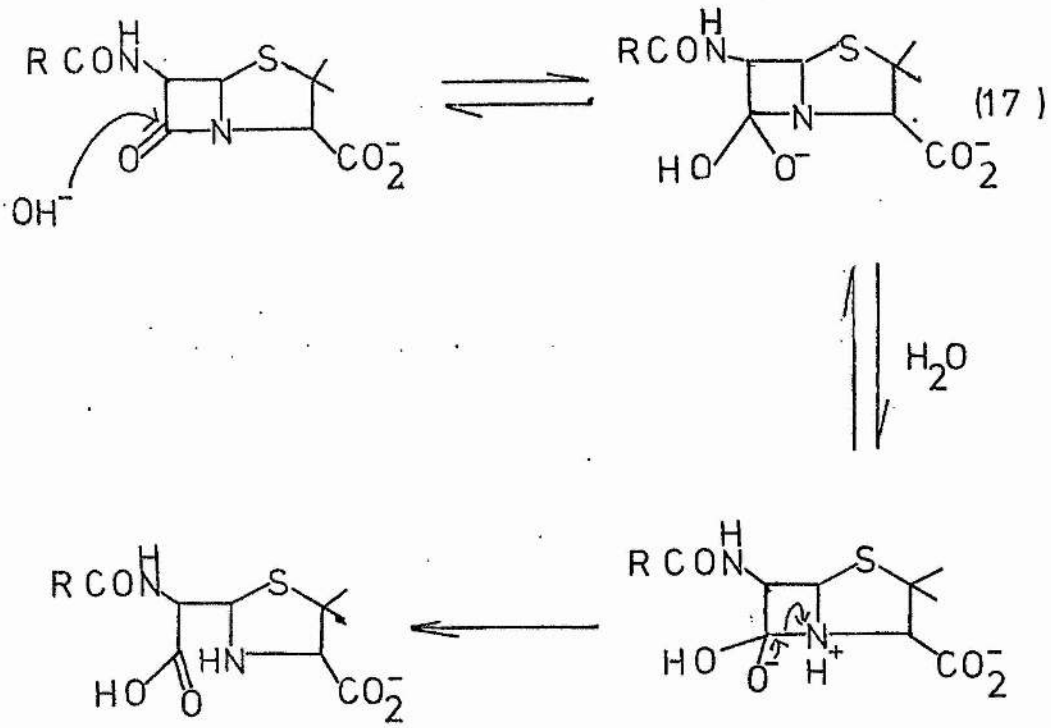
The experimental results were in good agreement with this relationship. It explains satisfactorily the kink which is observed in the pH - rate profile.

Brodersen also investigated the effect of temperature on the reaction, and calculated the energies of activation for the degradation of $HPnH^+$ and Pn^-H^+ to be 17.6 kcal/mol and 21.0 kcal/mol respectively.

Since Brodersen's work, investigators have tended to concentrate on the rates of formation of particular products. Krejci, using polarography, demonstrated the existence of a polarographically active intermediate which was formed in the course of the acid-inactivation of benzylpenicillin. By comparison with ultraviolet studies, he concluded that this intermediate was the penicillenic acid, and elucidated the rate constants for its formation and decay[41]. His results indicated that the proportion of penicillenic acid formed, relative to the total product, increases with increasing pH. Later, Schwartz, combining Krejci's results with Brodersen's, concluded that the penicillenic acid was formed by reaction of the penicillin anion (Pn^-H^+), while the undissociated penicillin ($HPnH^+$) reacted to form penicilloic acid[42]. (This is a conclusion which will be criticized later in the thesis.)

Dennen and Davis[43] investigated the formation of penicilloic acid from a number of penicillins, detecting these products by means of arsenomolybdic acid and mercuric chloride. Their results indicate that the proportion of this product formed, relative to the total product, decreases as the pH of the reaction medium is increased.

More recently, studies have been carried out in which the entire range of possible products has been monitored simultaneously as a



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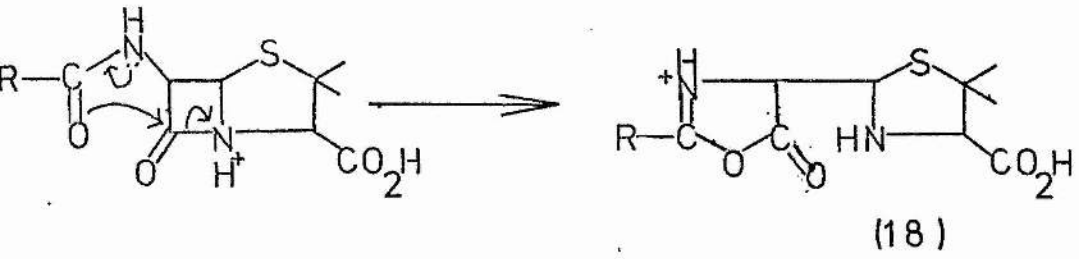

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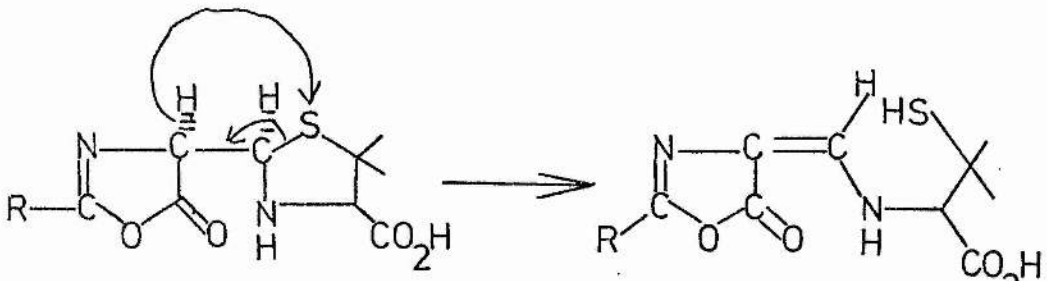
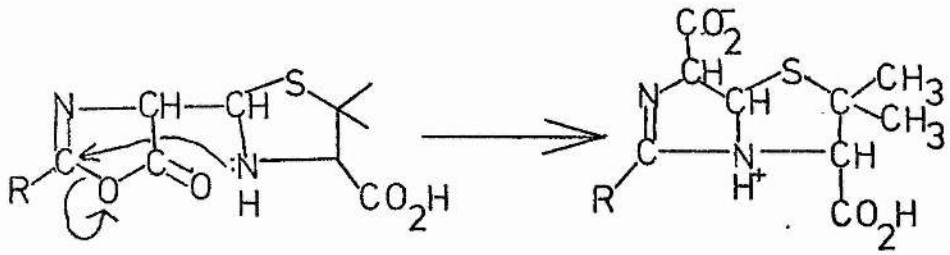


(18) penicillenic acid

SCHEME 5



(18) penillic acid

SCHEME 6

function of time. Methods employed for this have been High Pressure Liquid Chromatography[32] and High Resolution Fourier Transform NMR spectroscopy[44].

In alkaline solution, where the reaction is much more straightforward, it has been established that the rate of reaction is first-order with respect to both benzylpenicillin and hydroxide ion[45].

It is intuitively easy to presume that the alkaline inactivation of penicillin proceeds via nucleophilic attack of hydroxide ion on the β-lactam carbonyl (scheme 3). Both the identity of the product and the kinetics of the reaction would support this hypothesis. Recently, it has been demonstrated[46] that aminolysis reactions proceed in the same manner, and that the rate-limiting process in such cases is the formation of the tetrahedral intermediate analogous to (17).

In contrast to this simple situation, the observed isomerisations into penillic and penicillenic acids, which occur in acidic media, must involve more complex mechanisms. Interestingly, the only mechanisms proposed for these transformations to date were postulated considerably in advance of most kinetic work[47].

Both these mechanisms involve an initial protonation of the β-lactam nitrogen, just as Brodersen envisaged. The C.- N bond is then cleaved via an intramolecular nucleophilic attack from the side-chain carbonyl (scheme 4).

The intermediate which results from this, with the oxazolone-thiazolidine structure (18), was one of the original suggestions for the structure of penicillin itself. However, attempts to synthesize such a compound demonstrated that it was far too unstable. For example, a simple hydride shift will convert it into a much more stable structure: penicillenic acid (scheme 5).

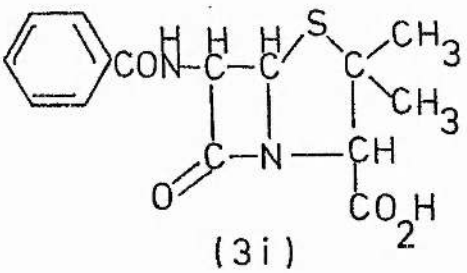
This intermediate (18) may also be converted into penillic acid by means of a further intramolecular nucleophilic attack. (Scheme 6). It should be noted here that the interacting centres are not actually so far apart as they appear on paper.

Significant evidence has been collected in support of these mechanisms, both by the present author and by other researchers. This will all be presented in the main body of the thesis.

This, then is a very brief summary of what has been learned about penicillin reactions in the fifty years since the initial discovery. What follows, though it occupies many more pages, represents by comparison a very tiny advance.

CHAPTER TWO

SYNTHESIS OF PENICILLINS AND RELATED COMPOUNDS



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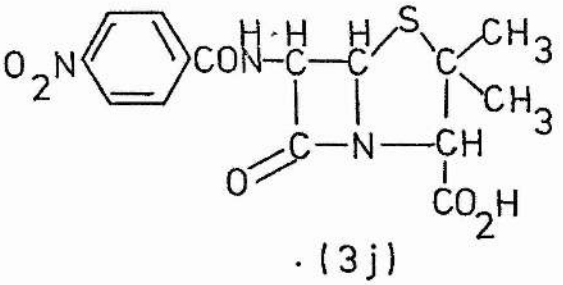
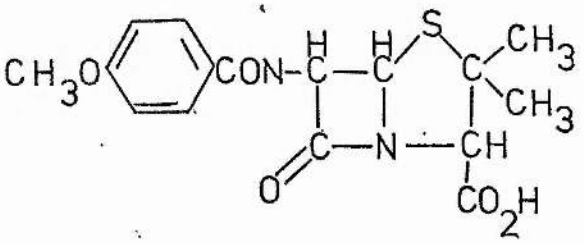
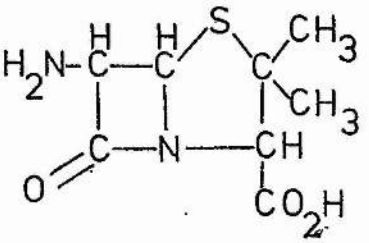


(3j)



(3k)



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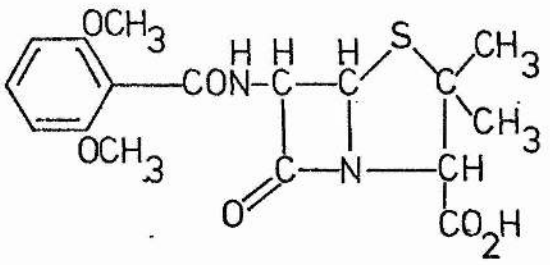

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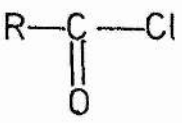

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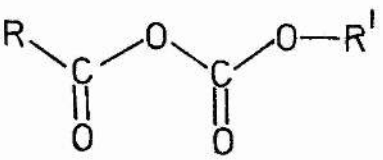

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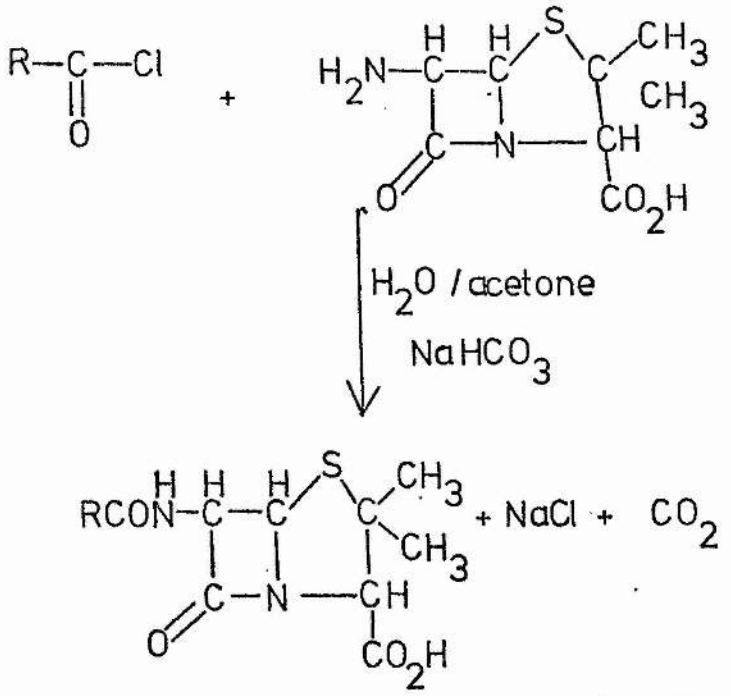
INTRODUCTION AND DISCUSSION

A. SEMISYNTHETIC PENICILLINS

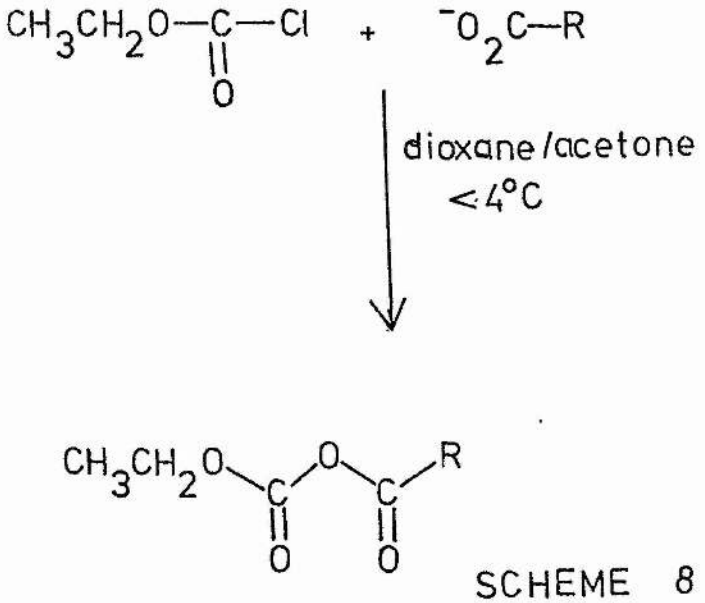
The many complicated pathways by which benzylpenicillin reacts in dilute acid have been the subject of much research, the main conclusions of which were summarised in the previous chapter. Further work on this aspect, conducted as part of the present project, is communicated in chapter 3. However, one of the principal objectives of this project has been to try to see if modifications of the penicillin side-chain would alter the reaction scheme. It was decided to investigate the reaction of phenylpenicillin (3i), p-nitrophenylpenicillin (3j) and p-methoxyphenylpenicillin (3k). These three form a series in which the substituents have contrasting electronic properties, the methoxy group having a strongly activating effect on a benzene ring while the nitro group is strongly deactivating.

Phenylpenicillins have not been found to be very efficacious as antibiotics. The only member of the series used in medicine is methicillin (3f), or 2,6-dimethoxyphenylpenicillin. The three compounds chosen for study, therefore, were not available commercially and so required to be specially synthesised.

The isolation of 6-aminopenicillanic acid, or 6-APA (1), has opened the way to the synthesis of penicillins with an extensive variety of side-chains. In theory, all that is required is to prepare the desired side-chain in the form of a suitably activated carboxylic acid, which can then be condensed with the primary amine group of 6-APA.



SCHEME 7



SCHEME 8

Two types of activated carboxylic acid have been found useful in the acylation of 6-APA. These are acyl chlorides (19) and mixed anhydride reagents (20).

A large number of preparations of semisynthetic penicillins using acid chlorides were reported by Nayler and co-workers [11, 13, 48] shortly after their isolation of 6-APA. The condensations can be effected in aqueous solution using modified Schotten-Baumann conditions, where sodium bicarbonate is used to neutralise the 6-APA and the nascent HCl, without making the medium so alkaline that the β-lactam function is destroyed. An example of this approach is the preparation of triphenylmethylpenicillin[48]. (Scheme 7)

Where the appropriate acid chloride is particularly sensitive to water, it may be possible to carry out the reaction in an organic solvent such as chloroform, using triethylamine as a base. The problem with this method is the poor solubility of 6-APA free acid in organic solvents. It is only really soluble as a salt in water. However, the method has been used in the preparation of methicillin[13].

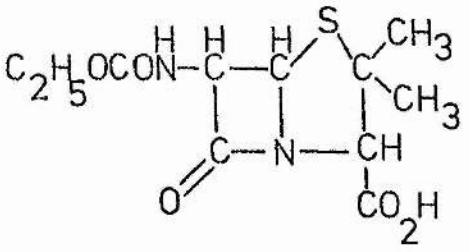
The mixed anhydride method was described by Perron et al.[49], and used, for example, in the preparation of 4-acetamidophenoxyethylpenicillin. The mixed anhydride reagent is prepared by condensing the triethylamine salt of the appropriate carboxylic acid with ethyl or isobutyl chloroformate at low temperature (scheme 8). The product is extremely effective as an acylating agent for amines.

In practice it was found that, of the compounds whose preparation was desired, the most easily accessible was p-nitrophenylpenicillin. This is formed by condensation of 6-APA with p-nitrobenzoyl chloride in aqueous sodium bicarbonate and acetone[50]. The product is a mixture of four substances, one of which is the desired penicillin. The

penicillin can be separated from the rest, either by column chromatography, or by the following procedure. The crude product was acidified and extracted into isobutyl methyl ketone. Addition of a solution of potassium 2-ethylhexanoate caused precipitation of the pure penicillin as its potassium salt[49]. All the preparations of this compound contained a molecule of water; it proved impossible to form a completely anhydrous penicillin salt.

Samples of phenylpenicillin were prepared by both the acid chloride method and the mixed anhydride method, both of which appeared to be equally efficacious. However, in this case the isolation of pure penicillin from the crude product was not so simple. A specific method for the preparation of phenylpenicillin is described in the literature[51], but this did not lead to a pure product. Column chromatography failed to separate the components of the mixture. The product was acidified as before and extracted into isobutyl methyl ketone. Addition of potassium 2-ethylhexanoate this time did not cause precipitation, but the subsequent addition of a large volume of ether did. This white precipitate proved to be a mixture of the penicillin salt and potassium benzoate, which could be separated by taking up the former in acetone. Again, only the hydrated penicillin was isolated. The yield of phenylpenicillin was rather less than could be obtained for p-nitrophenylpenicillin.

The preparation of p-methoxyphenylpenicillin proved to be extremely troublesome. The first attempt was an aqueous condensation of 6-APA with anisoyl chloride - an approach which had been successful in the previous two cases. However, this time the major product was simply anisic acid. It appears that anisoyl chloride, the least reactive of the three chlorides, is far more reactive towards water than



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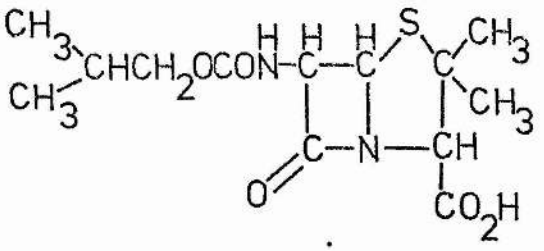

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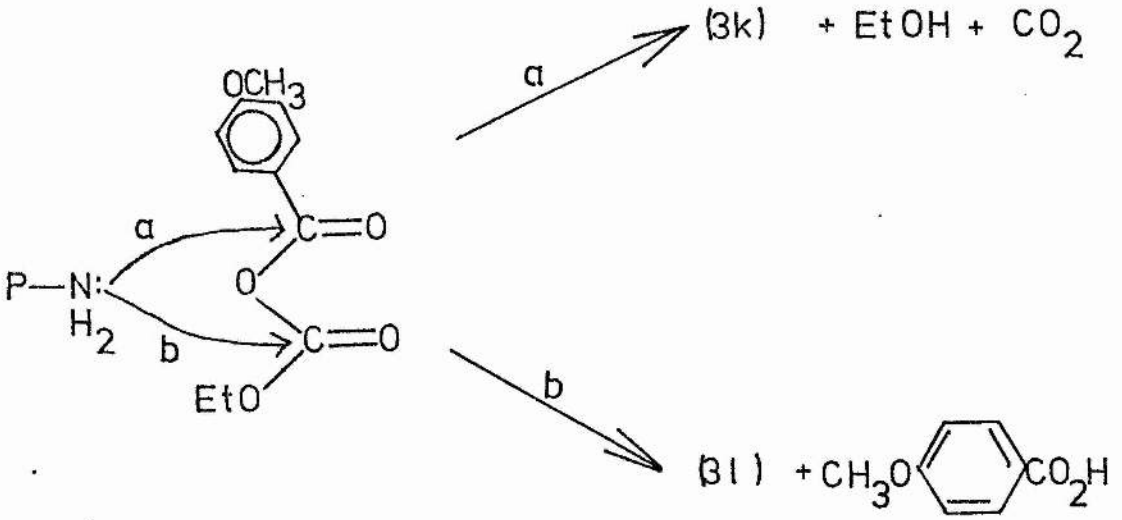


SCHEME 9

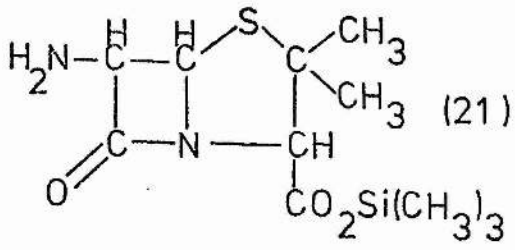
towards 6-APA. The mixed anhydride method was tried next. This did lead to the formation of penicillin, but the desired p-methoxyphenyl-penicillin was found to be mixed with large amounts of ethoxypenicillin (3l) (or isobutoxypenicillin (3m)). Scheme 9 represents how these contaminants can arise. The p-methoxy group renders the phenyl carbonyl less susceptible than usual to nucleophilic attack, and so encourages attack by the amine on the other carbonyl. It proved impossible to separate this mixture of penicillin by a simple method.

Thirdly, anisoyl chloride was tried again, but this time the reaction was performed in dry alcohol-free chloroform. However, no penicillin could be isolated.

Success was finally attained when the 6-APA was rendered soluble by converting it into its trimethylsilyl ester (21). This reacted easily with anisoyl chloride in dioxane/chloroform, and the ester was hydrolysed by addition of aqueous ethanol[52]. Even by this method, though, the yield of p-methoxyphenylpenicillin was very small, and the product hydrolysed very easily.

In the end, samples of all three compounds were obtained. Spectral studies and elemental analysis confirmed their identity and purity, so that kinetic studies were able to go ahead. These are described in the next chapter.

There are three important spectral characteristics of penicillins which were used extensively in the characterisation of these compounds. In the infra-red spectrum the β-lactam stretching frequency occurs at 1770-1780 cm^{-1}. In the NMR spectrum the two resonances are rather close together, occuring at about 1.5 ppm and separated by about 0.1 ppm. Penicillins themselves have unremarkable UV spectra, but on standing in dilute acid they begin to absorb strongly above



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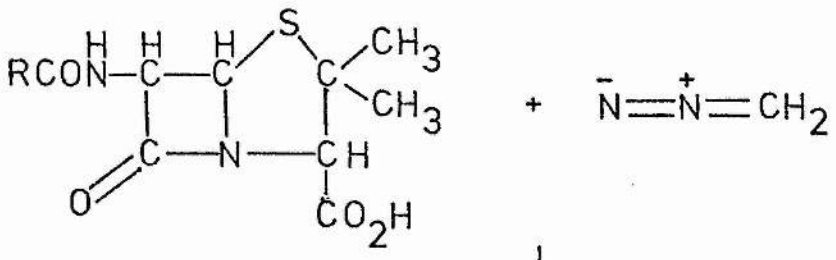

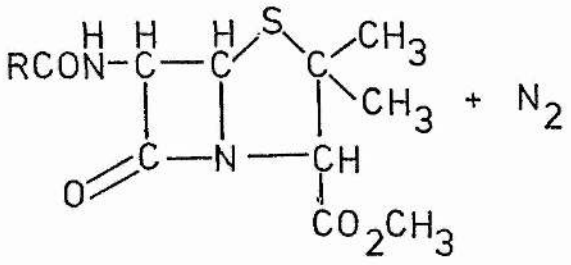

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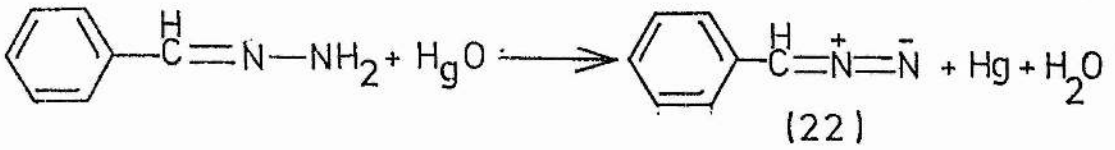

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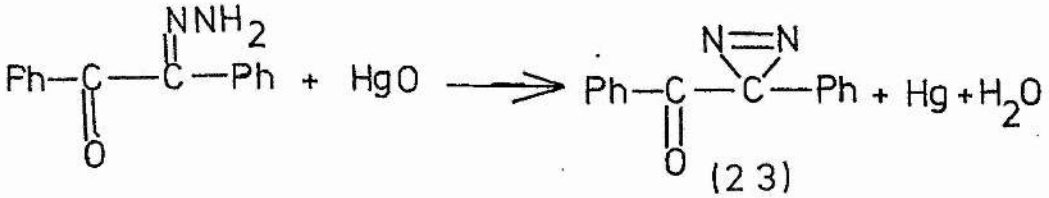

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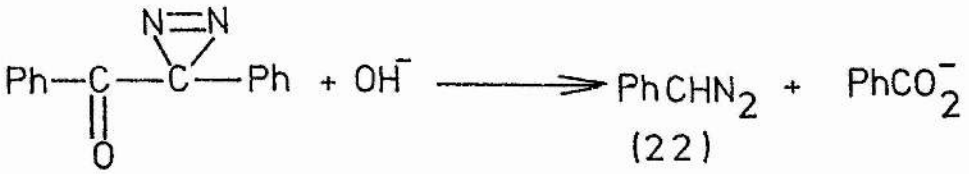


(22)

SCHEME 12

(24)

SCHEME 13

300 nm. In time this absorption reaches a maximum and then begins to decline. It is associated with the formation of penicillenic acid.

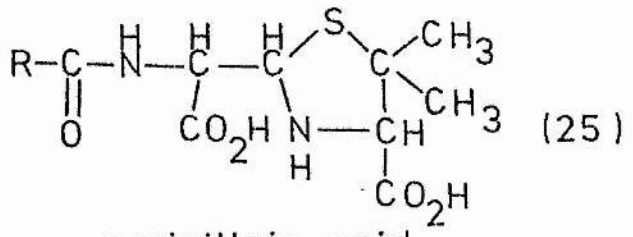
B ESTERS

There are two reasons why it was desired to prepare penicillin esters during this project. Firstly, methyl esters were prepared in order to compare their rates of reaction with those of the unesterified penicillins. Secondly, some benzyl-type esters were required as intermediates for further chemical reactions.

Methyl, benzyl and benzhydral esters were prepared by reaction of substituted diazomethanes on the penicillin (scheme 10)[53]. p-Nitrobenzyl esters were formed by the action on penicillin of p-nitrobenzyl bromide[54].

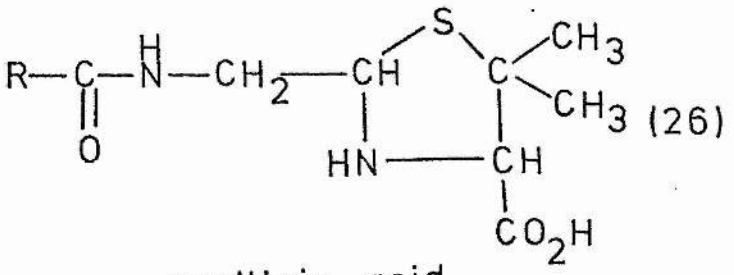
Many attempts were made to esterify 6-APA; but all of these, with the exception of the trimethylsilylation mentioned above, proved unsuccessful. The literature does record a method for the preparation of 6-APA benzyl ester[55], but even with this it was found impossible to obtain satisfactory results. In contrast, the esterification of penicillins proceeds very smoothly and leads to pure products.

Of the esterifying agents, diazomethane is easily prepared from a standard precursor, N-methyl-N-nitrosotoluene-p-sulphonamide[56]. Two routes were employed to prepare phenyldiazomethane (22). The first of these was by the oxidation of benzaldehyde hydrazone using yellow mercuric oxide (scheme 11). The second involved the preparation, as an intermediate, of azibenzil (23) - by the oxidation of benzil monohydrazone[57]. This intermediate can be stored for months, and is easily converted to phenyldiazomethane by the action of strong alkali (scheme 12)[58]. This second method became the preferred one, since it is



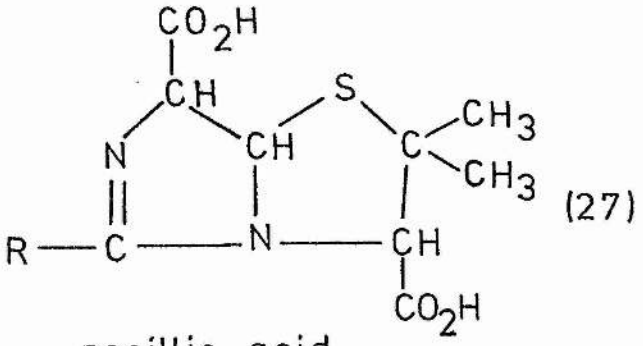
(25)

penicilloic acid



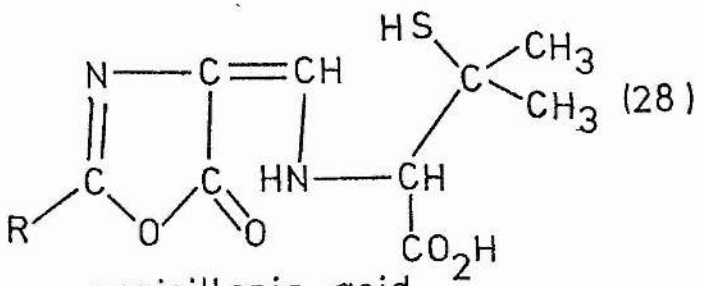
(26)

penilloic acid



(27)

penillic acid



(28)

penicillenic acid

more convenient and gives a purer product. Diphenyldiazomethane (24) was prepared by the oxidation of benzophenone hydrazone (scheme 13)[59].

C DEGRADATION PRODUCTS OF THE PENICILLINS

Having prepared the semisynthetic penicillins, it was also important to obtain authentic samples of the compounds they might possibly be transformed to during their reactions. This is necessary if the courses of their reactions are to be accurately charted. By analogy with benzylpenicillin, the degradation products likely to be of interest are penicilloic acids (25), penilloic acids (26), penillic acids (27) and penicillenic acids (28).

Penicilloic acids (25) are the easiest of these compounds to prepare. They arise from the action of dilute alkali on the penicillin[30]. All three semisynthetic penicillins have been converted to their penicilloic acids.

Benzylpenilloic acid (26, R = $PhCH_2$-) may be prepared most simply by refluxing the penicillin with dilute HCl[30]. From the mixture, pure penillic acid may be easily isolated. This is not the case with the phenylpenicillins, and here it was found preferable to treat the penicilloic acid with HCl. At room temperature, penilloic acid forms in about a week; but the reactions may be performed at elevated temperatures.

Again in the case of the penillic acids (27), only the benzyl compound can be easily isolated in a pure form. Pure penillic acid precipitates from a solution of benzylpenicillin in HCl[31]. This did not happen in the case of phenylpenillic acid, where it was found necessary to isolate the desired product from a mixture by means of preparative thin layer chromatography. This compound has still not been well characterised, because only a tiny amount of pure material has been obtained. Indeed, the only evidence that the product is a penillic acid is by analogy with

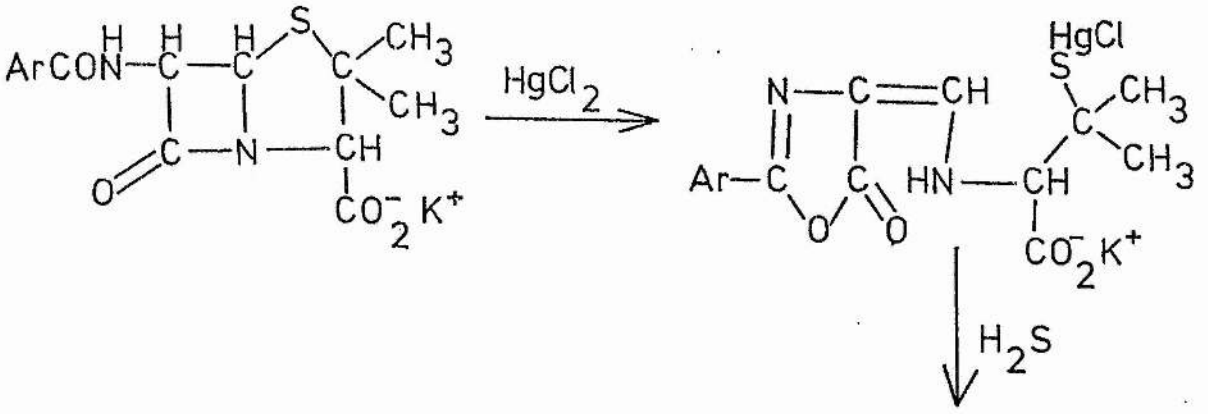
the benzyl compound. It arises by the action of dilute acid on the penicillin; compared with other degradation products it has a low R_f value, and in its NMR spectrum the two $-CH_3$ resonances are closely spaced.

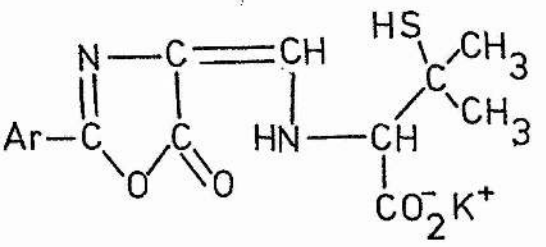
The most interesting of the degradation products, from the point of view of this study, were the penicillenic acids (28). In the studies of the acid-degradation of penicillins which were undertaken, it was the formation of penicillenic acids which was monitored, utilizing the intense UV absorption which is a common feature of these compounds. It is disappointing, therefore, that the attempted syntheses have not been greatly successful.

Two approaches to the synthesis of these compounds were adopted. In one, an aqueous solution of the penicillin was treated with mercuric chloride solution, causing precipitation of the penicillenic acid mercury mercaptide. From this, free penicillenic acid was released by the action of H_2S gas (scheme 14)[34]. The second approach was an attempt to build the penicillenic acid up from smaller units. Thus, reaction of the appropriately substituted hippuric acid (29) with acetic anhydride and triethyl orthoformate led to the formation of ethoxymethyleneoxazolones (30)[60, 61]. These were then condensed with D-penicillamine (15) to form the penicillenic acids (scheme 15)[62].

Both of these approaches have been described in the literature for benzylpenicillenic acid; and this compound can be prepared in good yield and purity. However, with the phenyl compounds, a mixture of at least two substances ensues. NMR and UV studies suggest that the same mixture is obtained by both synthetic routes.

Extinction coefficients for phenyl and p-nitrophenylpenicillenic acids have been measured by allowing the penicillins to react with an imidazole solution containing a tiny amount of mercury ion[75]; and the





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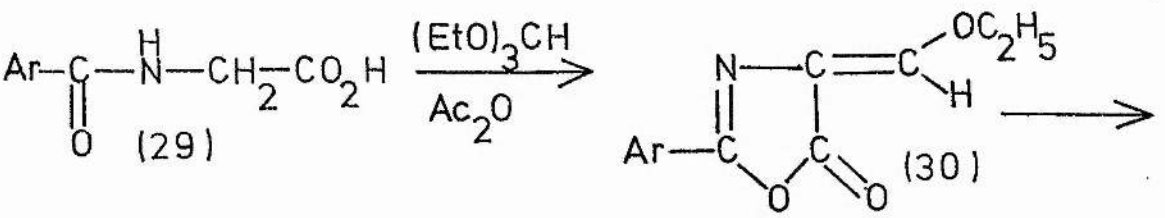

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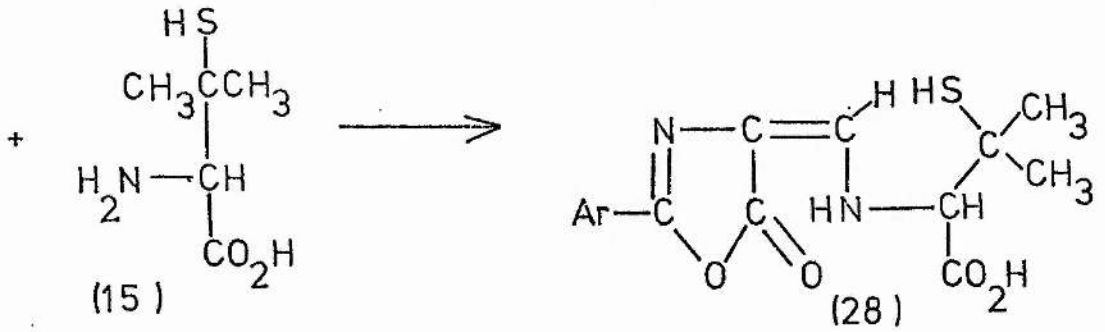

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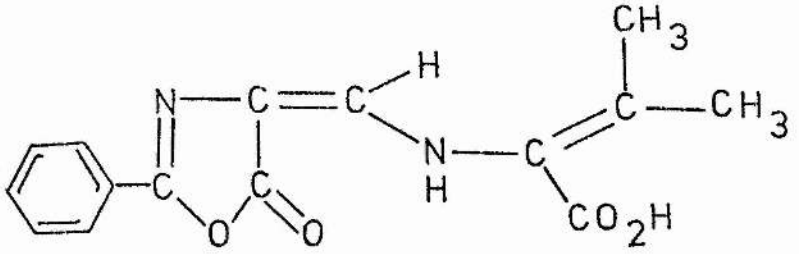
SCHEME 15



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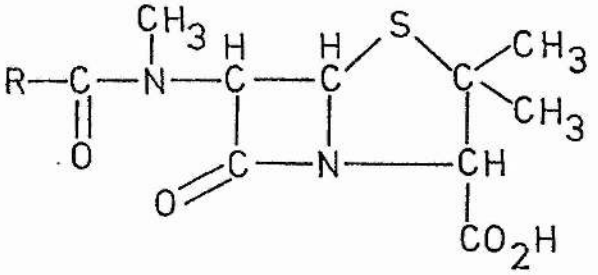


(35)

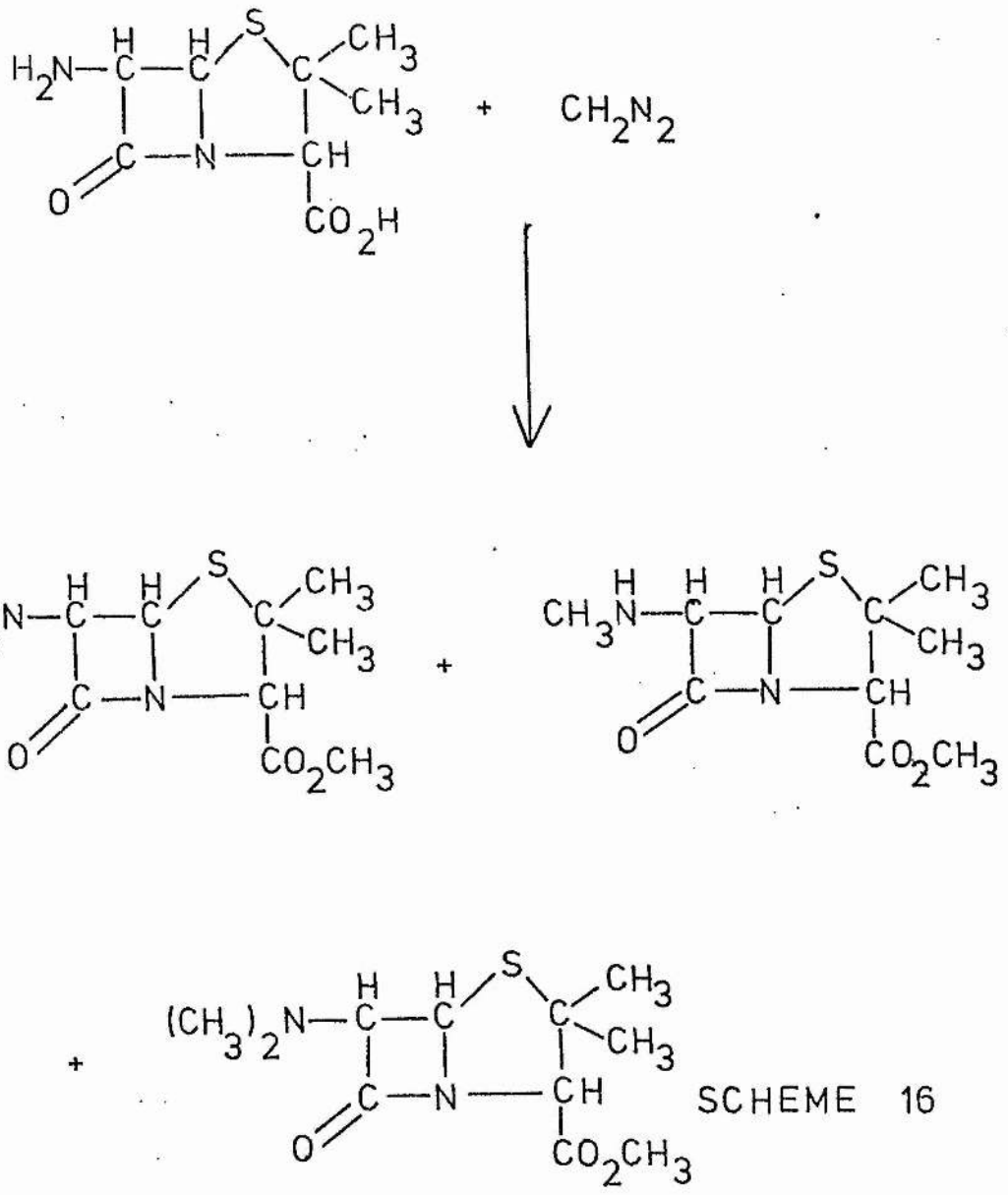
synthetic preparations had UV spectra which accorded well with these measurements. This is surprising, since any degradation product of penicillenic acid would be expected to have a far lower extinction coefficient. Elemental analysis of a phenylpenicillenic acid preparation indicated that the substance was considerably deficient in sulphur. Thus it may be postulated that the second component of the mixture arises by loss of H_2S from penicillenic acid, and has structure (31). Such a structure would be expected to have its extinction coefficient as high as for penicillenic acid itself. In its NMR spectrum, the methyl resonances would be widely spaced (1.30, 1.70 ppm); while for the penicillenic acid they would be closer (1.40, 1.50 ppm). This accounts for the observed NMR spectrum of the product.

Kinetic studies of the reactions of phenylpenicillins with dilute acid have indicated that their penicillenic acids behave in a radically different way from benzylpenicillenic acid (see chapter 3). Perhaps these synthetic anomalies are simply yet another example of this difference.

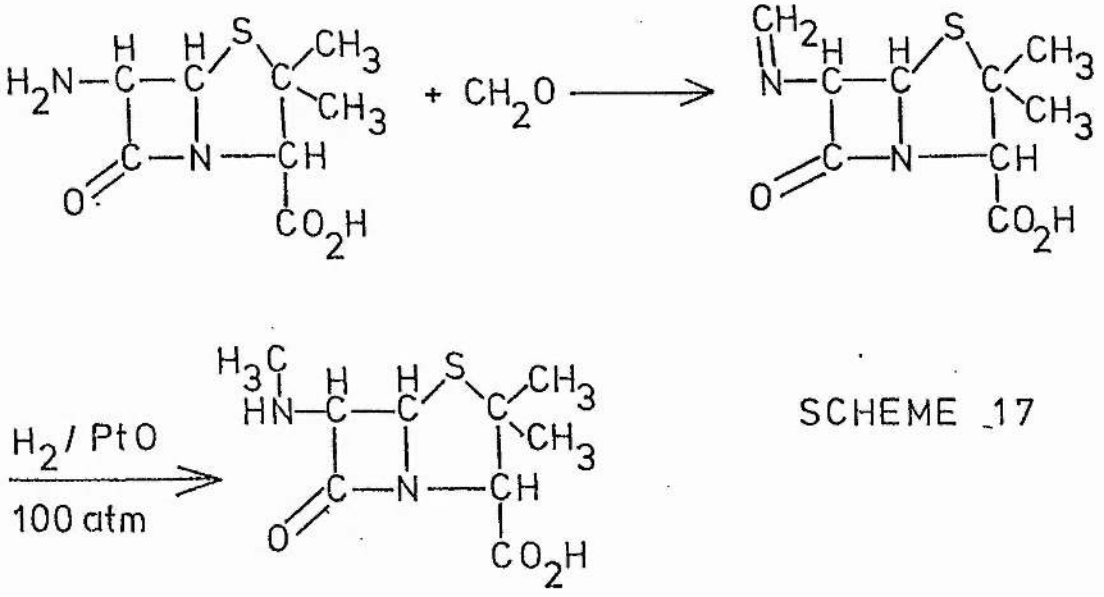
D N-METHYLPENICILLINS

In the study of the acid degradation of penicillins, it became apparent that an important step in the mechanism was the removal of a proton from the 6-amido function. This is necessary for the formation of penillic acids and of penicillenic acids. It was felt that it would be interesting to see what happens when there is no proton to be removed from that position. To this end, it was desired to prepare a series of N-methyl penicillins (35), for comparison with the unmethylated compounds.

The literature gives details of two methods for N-methylating penicillins, neither of which proceed with very good yields. In the first method, 6-APA is treated with a large excess of diazomethane[63].



SCHEME 16



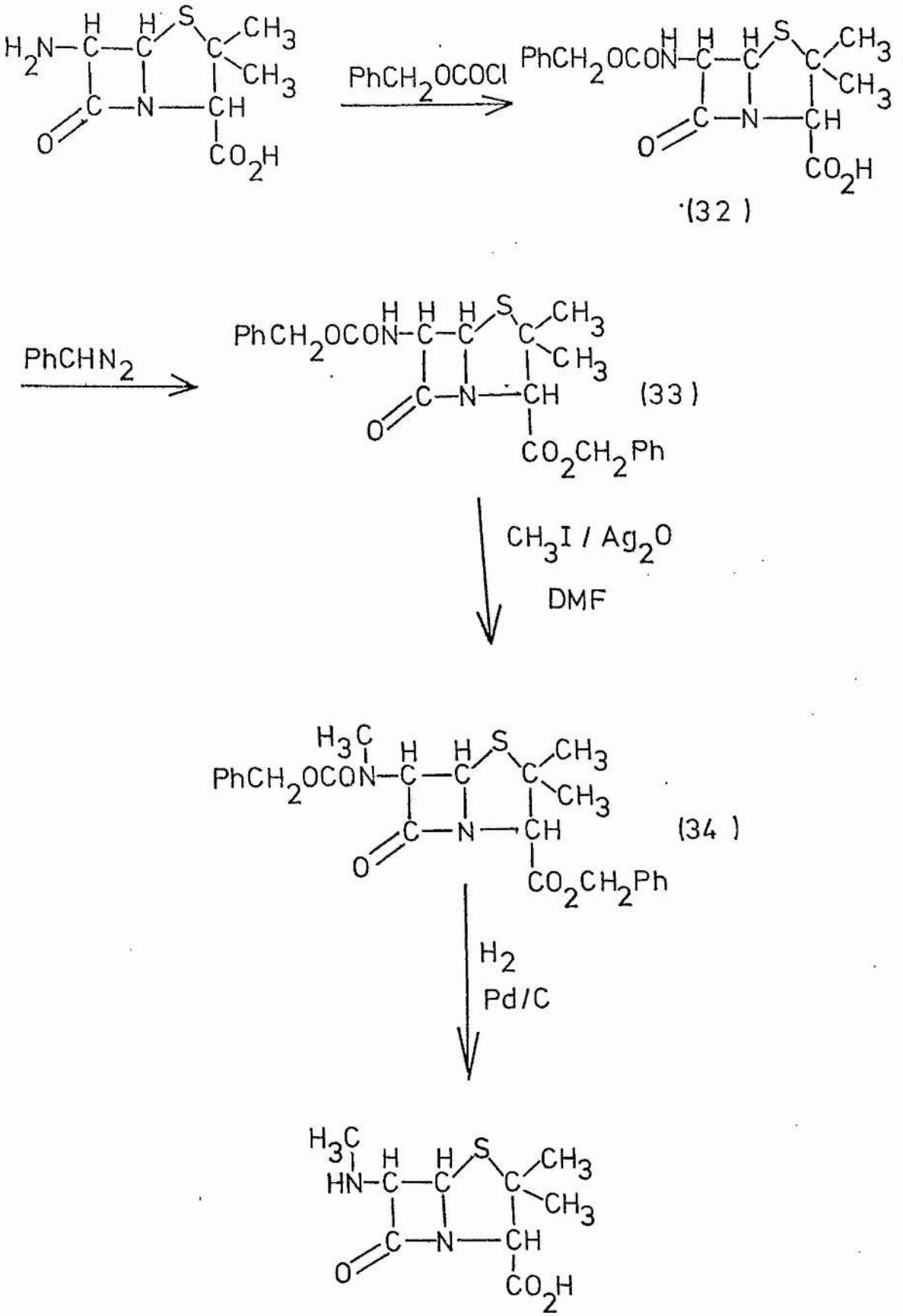
SCHEME 17

The result is a mixture of 6-APA methyl ester, N-methyl-6-APA methyl ester and N,N-dimethyl-6-APA methyl ester (scheme 16). These can be separated by column chromatography. The monomethyl derivative can then be acylated in the usual manner to give N-methylpenicillin methyl esters. The overall yield at this point is about 7%. The real difficulty, though, arises on attempting to saponify the ester, since the conditions required for this lead to cleavage of the β-lactam ring. After saponification, the amount of intact penicillin in the mixture is only about 1%, and it is impossible to separate this from the hydrolysed material. So the desired product cannot be obtained pure.

It is known that diazomethane will only methylate acidic functions; and in the above case the nitrogen is only methylated because some of the 6-APA exists in the zwitterionic form. Thus it would serve no purpose to perform the experiment on a 6-APA benzyl ester (thus rendering de-esterification more facile) because then the nitrogen could never be acidic enough to react with diazomethane.

The other method reported in the literature involves a reductive condensation of 6-APA with formaldehyde, followed by acylation with an acid chloride (scheme 17)[64]. The overall yield of this process is only 5%. Since the method requires very high pressures and very expensive reagents, it was decided not to attempt it.

It was felt that it ought to be possible to methylate penicillins more efficiently than this; and the method outlined in scheme 18 was devised. This based on Olsen's methylation of protected amino-acids, using methyl iodide and silver oxide in dimethylformamide solvent[65]. The carboxyl and amino functions of 6-APA are first protected by benzyl and benzyloxycarbonyl groups respectively. It was thought that these would be easily removable after the methylation step. The product,



SCHEME 18

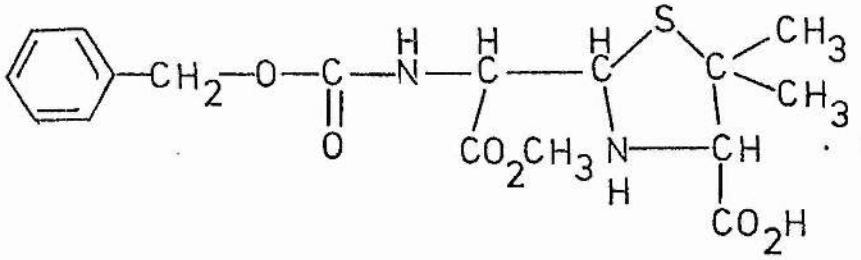
6-methylaminopenicillanic acid, could then be acylated in the usual fashion.

In practice, acylation of 6-APA with benzyl chloroformate proceeded smoothly and in good yield, forming N-benzyloxycarbonyl-6-APA (32 Treatment with phenyldiazomethane then afforded the benzyl ester (33), which could be methylated with ease, giving N-benzyloxycarbonyl-N-methyl-6-APA benzyl ester (34). The difficulty arose on trying to remove the protecting groups from this compound; and at the time of writing this difficulty has yet to be overcome.

It was found that the benzyl ester group could be cleaved off by catalytic hydrogenation at 45 psi, in a mixture of methanol and sodium bicarbonate solution[66]. This treatment, however, did not cleave off the N-benzyloxycarbonyl group. Hydrogenolysis of (32) and (33) thus gave rise to the same product, which was identified as N-benzyloxycarbonyl-6-aminopenicilloic acid methyl ester (36). Prolonged exposure to methanol leads to cleavage of the β-lactam ring and formation of this ester. This cleavage can be avoided by removing the solvent immediately. Thus, taking this precaution, hydrogenolysis of (34) gave (37).

Olsen[66] has reported that N-benzyloxycarbonyl groups may be cleaved off by hydrogenolysis in an acidic medium. In the present case, however, his method was unsuccessful. Neither the benzyl nor the benzyloxycarbonyl group was removed; but there was evidence that methanolysis of the β-lactam had taken place, despite the fact that the solvent was removed with all practicable speed.

This resistance of the benzyloxycarbonyl group to hydrogenolysis was a problem entirely unforseen at the commencement of the synthesis. When the difficulty became known, some ways were sought of avoiding the use of this protecting group.



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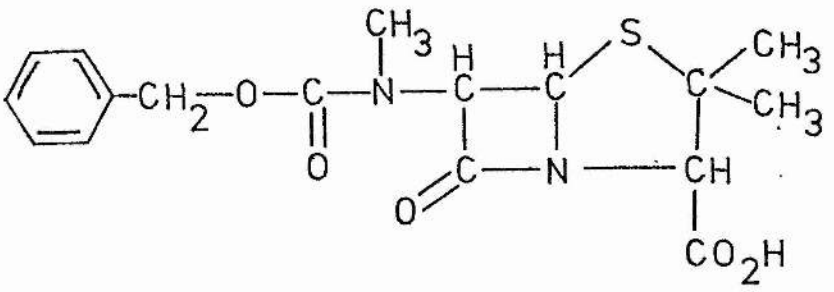

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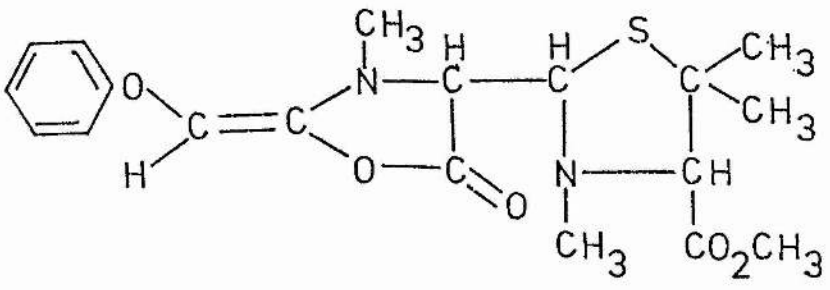


(38)

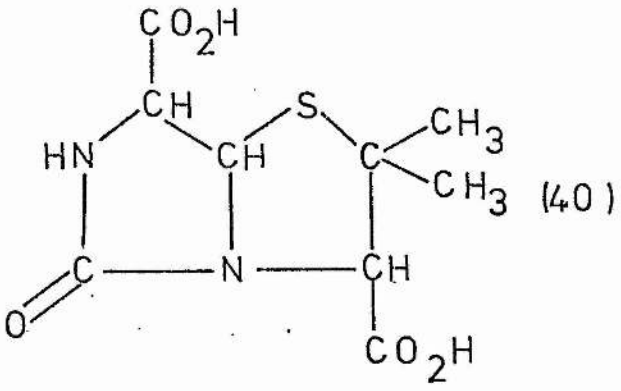
Since the methylation of benzyloxycarbonyl-6-APA benzyl ester proceeded so smoothly, it was decided to examine the effect of this process on genuine penicillins.

With benzylpenicillin, the CH_3I/Ag_2O reagent did react. This could be deduced from the appearance of silver iodide as a byproduct. However, in the process, the β-lactam ring was cleaved (there was evidence for this from IR studies) and a complicated mixture of products was formed.

Rather better results were obtained with phenoxymethylpenicillin, (or penicillin V (3b)). Again, a mixture of products was obtained, but, from the NMR spectrum, 50% of it could be identified as N-methylpenicillin V methyl ester, with its β-lactam ring intact. The remainder of the mixture has not been positively identified, but a major component may be of structure (38). This can be envisaged as arising from electophilic attack of the methylating agent on the β-lactam nitrogen. Such a structure would explain the relatively low intensity of the $-CH_2-$ and C_5/C_6 resonances in the NMR, and also account for the rather high resonances at 8.00 ppm. This, however, is just speculation.

The problem here seems to be that the methylating agent attacks not only the side-chain amide, but also the β-lactam nitrogen, causing cleavage of the ring. The ease with which this takes place seems to parallel the ease of reaction with acids: ie penicillin G is much more susceptible than penicillin V, and benzyloxycarbonyl-6-APA (32) is not affected at all at its β-lactam function. (The differing susceptibilities of penicillins to electrophilic reagents is a topic which will be discussed in chapter 3.)

After the failure of hydrogenolysis to remove the benzyloxycarbonyl group, other methods of doing this were sought. Particular attention



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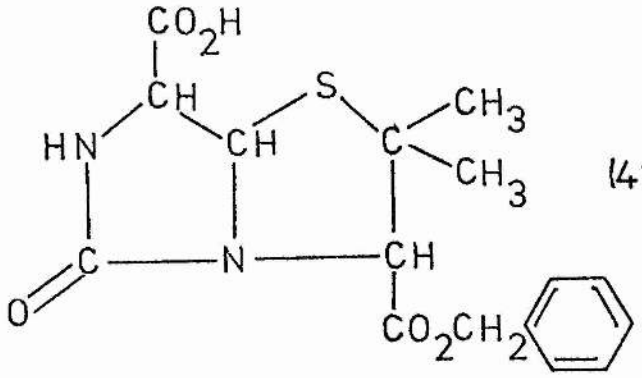


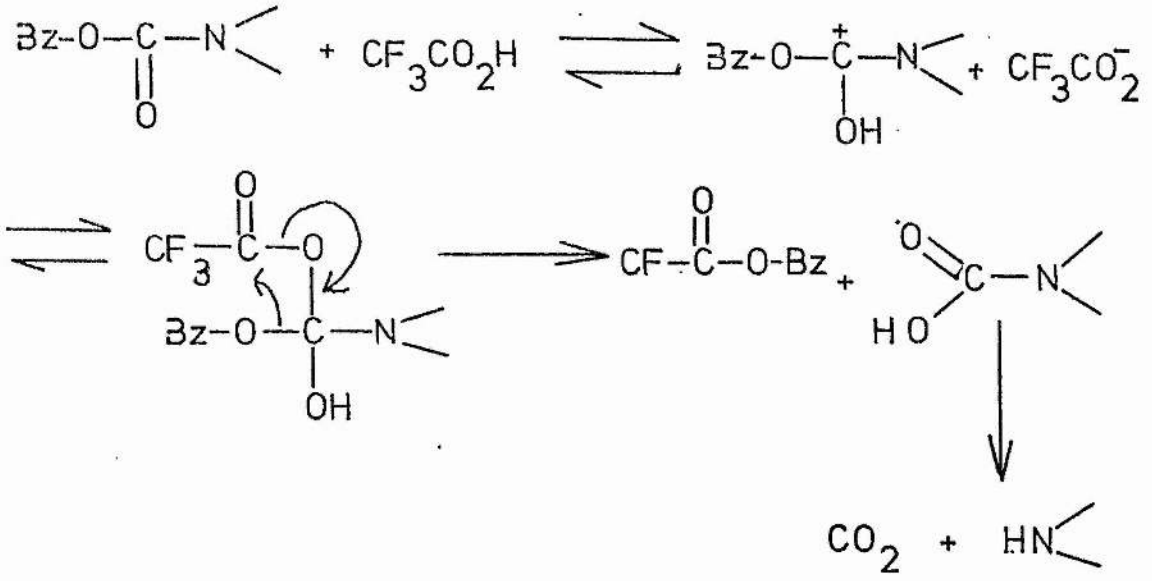
(41)

was given to strong acid reagents, such as HBr/CH_3COOH and trifluoroacetic acid. It was reasoned that the β-lactam of the substrate might well survive exposure to strong acid, as dilute acid had been observed to have no effect on benzyloxycarbonyl-6-APA (32). In contrast, strongly basic reagents such as sodium in liquid ammonia would be sure to destroy the ring.

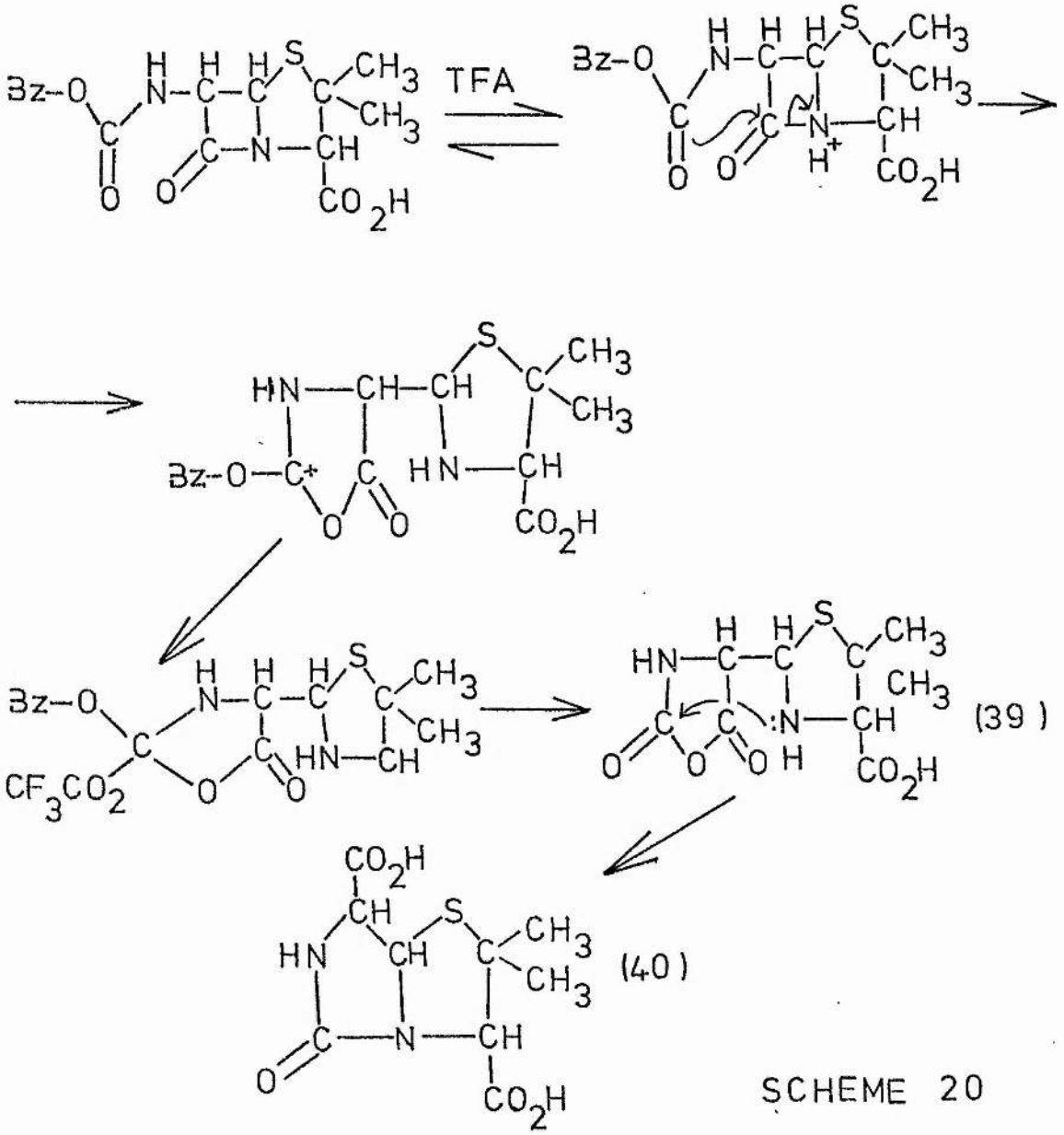
The use of trifluoroacetic acid to remove a benzhydral ester group from cephalosporin has been reported[68]. It was decided to investigate the effect of this reagent on compound (34); but first the effect on some of its precursors was observed.

When 6-APA was treated with trifloroacetic acid, the β-lactam ring was lost, but no firm conclusion has been reached about the identity of the product. Treatment of benzylpenicillin with TFA also results in loss of the β-lactam.

Treatment of benzyloxycarbonyl-6-APA (32) with TFA results in removal of the side-chain as well as β-lactam cleavage. This was found to be a remarkably clean reaction, and it resulted in just one product. The side-chain, however, was transformed to benzyl alcohol, which contaminated the product and proved exceedingly difficult to remove. For this reason, a good elemental analysis has not been obtained. However, taking the contaminant into account, the formula appeared to be $C_9H_{12}N_2O_5S$; and there is good evidence that the structure is (40). This is similar to the penillic acid structure (27); and, as in the case of penillic acid, the $-CH_3$ resonances in the NMR spectrum are closely spaced. Also, the C_2 and C_6 protons have the same chemical shift. Treatment with strong alkali had no effect on this product. In its mass spectrum, the highest peak was at 138, which can be attributed to structure (40) minus its two carboxylic acid groups and the sulphur.



SCHEME 19



(39)

(40)

SCHEME 20

Treatment of N-benzyloxycarbonyl-6-APA benzyl ester (33) with TFA also gave a single product, which was identified as (41), the monobenzyl ester of (40). The TFA did not cleave off the ester group. Again, benzyl alcohol proved to be an intractable contaminant.

Some references to a compound of structure (40) have appeared before in the literature [69, 70, 71], but it has not been properly characterised before now.

Some words concerning mechanism are pertinant at this stage. In divesting ordinary amines of their benzyloxycarbonyl protecting groups, TFA acts as shown in scheme 19, the free amine arising from decomposition of an intermediate carbamic acid. It is thought that in the case of 6-APA, the initial protonation occurs on the β-lactam nitrogen (scheme 20). An intramolecular reaction then results in the formation of the cyclic anhydride (39). This in turn rearranges, by a process analogous to the penillic acid rearrangement[47] (scheme 6, page 9).

The isolation of this novel compound has been an interesting side-line; but the central problem of removing the benzyloxycarbonyl side-chain without destroying the β-lactam function remains unsolved. It appears that all of the normal chemical techniques have been exhausted; it may be that the answer lies with an enzymatic cleavage process, similar to that used to obtain 6-APA from benzylpenicillin[8].

EXPERIMENTAL

All of the materials used in the preparations were of general reagent grade. The melting points quoted are uncorrected. UV spectra were recorded on a Pye-Unicam SP8-100 spectrophotometer; NMR spectra were

recorded on a Brucker WP80 instrument; IR spectra were recorded on a Perkin-Elmer 257 spectrophotometer, samples being prepared in the form of a disc with potassium bromide.

CHROMATOGRAPHY

It was felt desirable to find a chromatography solvent system which would effectively discriminate between a penicillin and its several degradation products. Based on a report of Vandamme and Voets[72], the following experiments were preformed.

Five penicillins were investigated by four TLC solvent systems. The compounds studied were penicillin G potassium salt, penicillin V free acid, methicillin sodium salt, ampicillin free acid and 6-amino-penicillanic acid. All were commercial preparations. Each substance (4 mg) was dissolved in a phosphate buffer solution (2 ml) of pH 7.42.

In addition to these neutral solutions, samples which had been aged in alkali and in acid were prepared. Thus each penicillin (8 mg) was dissolved in 0.1M NaOH solution (1 ml) and left to stand for one hour. Each solution was then neutralised with 0.1M HCl (1 ml) and the phosphate buffer solution (2 ml). Further samples of penicillin (8 mg) were dissolved in 0.1M HCl (1 ml) and allowed to stand for four hours. They were subsequently neutralised with 0.1M NaOH (1 ml) and the phosphate buffer (2 ml).

Each solution was spotted onto commercial plastic POLYGRAM TLC plates coated with silica gel to a thickness of 0.25 mm. The chromatograms were run in a Shandon TLC Chromatank, lined with Whatman no. 1 filter paper which had been previously saturated with the solvent. The solvent was allowed to rise a distance of 14 cm from the point where the sample was spotted.

The four solvent systems used were:

A. n-butanol/water/ethanol/acetic acid (5:2:2:1)

B. n-butanol/water/acetic acid (4:1:1)

C. acetone/acetic acid (95:5)

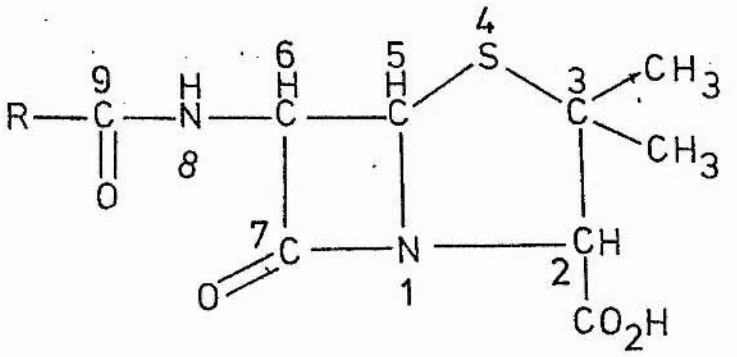
D. acetone/water (85:15)

After development, the solvent was allowed to evaporate from the plates, and visualisation of the spots was achieved by exposing the plate to iodine vapour in a chromatank.

The R_f values were as follows:

	A	B	C	D
Penicillin G	.72	.71	.78	.55
aged in alkali	.62	.54	(.11 - .38)	.34
aged in acid	.74,.61,.55	.69,.51,.39	.75,.19,.03	(.12 - .28),.
Penicillin V	.71	.69	.79	.57
aged in alkali	.63	.57	(.06 - .41)	.43
aged in acid	.70	.68	.75	.54
Methicillin	.69	.68	.74	.51
aged in alkali	.59	.47	(.04 - .49)	.34
aged in acid	.67,.56,.48	.66,.43,.25	.71,.19,.04	(.12 - .33)
Ampicillin	.60	.51	.68	.37
aged in alkali	.54	.35	.00	.20
aged in acid	.58	.48	.65	.36
6-APA	.50	.40	.59	.37
aged in alkali	.42	.28	.00	.14
aged in acid	.49	.38	.59	.36

The figures quoted in brackets represent streaks rather than discreet points.



reference diagram for NMR results

from these results it may be deduced that, of the five substances tested, only penicillin G and methicillin react easily with acids. They react to give at least two products, one of which seems to be the same as the product of reaction with alkali. All of the substances tested reacted with alkali. The product in each case is the penicilloic acid. Solvent B was chosen as the one which consistently gave the best separations. Throughout the following reports, unless otherwise stated, all chromatography results were obtained using this solvent system.

MATERIALS

Benzylpenicillin potassium salt. This was purchased from BDH company. IR: ν_{max} at 3370 cm^{-1} (N-H), 1770 cm^{-1} (β -lactam carbonyl), 1670 cm^{-1} (amide carbonyl), 1610 cm^{-1} (carboxylate group). NMR: (solvent D_2O); 1.50 ppm (s, $-CH_3$); 1.575 ppm (s, $-CH_3$); 3.65 ppm (s, $-CH_2-$); 4.25 ppm (s, C_2), 5.525 ppm (d, C_6), 5.45 ppm (d, C_5), 7.40 ppm (s, phenyl).

6-aminopenicillanic acid. This was purchased from the Sigma Company. Mp 207°C. IR: ν_{max} at 1770 cm^{-1} (β -lactam carbonyl), 1615 cm^{-1} (carboxylate group). NMR: (solvent D_2O/K_2CO_3); 1.55ppm (s, $-CH_3$); 1.60 ppm (s, $-CH_3$); 4.20 ppm (s, C_2); 4.60 ppm (d, C_6); 5.55 ppm (d, C_5).

Methicillin sodium salt. This was purchased from the Sigma Company. IR: ν_{max} at 3560 cm^{-1}, 3470 cm^{-1} (N-H), 1770 cm^{-1} (β -lactam carbonyl), 1670 cm^{-1} (amide carbonyl), 1600 cm^{-1} (carboxylate group). NMR: (solvent D_2O); 1.47 ppm (s, $-CH_3$); 1.58 ppm (s, $-CH_3$); 3.755 ppm (s, $-OCH_3$), 4.175 ppm (s, C_2); 5.50 ppm (d, C_5); 5.60 ppm (d, C_6); 6.70 ppm (d, phenyl); 7.40 ppm (t, phenyl).

Phenoxymethyl penicillin. This was purchased from the Sigma Company. IR: ν_{max} at 3320, 1750, 1655 cm^{-1}. NMR: (solvent acetone-d_6);

1.625 ppm (s, $-CH_3$); 1.675 ppm (s, $-CH_3$); 4.475 ppm (s, C_2); 4.70 ppm (s, $-CH_2-$); 5.775 ppm (m, C_5/C_6); 7.10 ppm (m, phenyl); 7.40 ppm (m, phenyl).

Ampicillin. This was purchased from the Sigma Company. IR: ν_{max} at 3500, 3430, 1775, 1680, 1600 cm^{-1}. NMR: (solvent D_2O); 1.45 ppm (s, $-CH_3$); 1.50 ppm (s, $-CH_3$); 3.90 ppm (s, C_9); 4.225 ppm (s, C_2); 5.475 ppm (d, C_5/C_6); 7.475 ppm (s, phenyl).

p-Nitrophenylpenicillin. a) By a method suggested by the preparation of triphenylmethylpenicillin[48]. A solution of p-nitrobenzoyl chloride (1.1g) in acetone (36 ml) was added to a solution of 6-APA (1.3g) in 3% aqueous sodium bicarbonate solution (50 ml) and acetone (15 ml). This mixture was stirred at room temperature for three hours. After washing with ether (2 x 60 ml), the aqueous solution was filtered and lyophilised. The deep yellow solid which remained was shaken with acetone (20 ml), and the insoluble part was filtered off. (This proved to be the excess sodium bicarbonate.) The yellow solution was evaporated, leaving a yellow powder (1.5g). TLC (on silica gel, with acetone /water -9:1) indicated the presence of four components. Separation was effected on a column of silica (50 cm x 2 cm) using the same solvent as elutant. 5 ml samples were collected from the bottom of the column, and analysed by TLC. Samples 4 - 21 contained just one component. They were pooled together and lyophilised. Yield 0.8g (32%). IR: ν_{max} at 1780, 1760, 1670, 1650, 1600cm^{-1} (Found: C, 43.19; H, 3.84; N, 9.95. $C_{15}H_{14}N_3O_6SNa.1.65\ H_2O$ requires C, 43.19; H, 4.18; N, 10.07%)
b) A modification of the method of Depue, Moat and Bondi[50]. 6-APA (1.08g) and sodium bicarbonate (2.1g) were dissolved in water (90 ml) and acetone (60 ml). p-Nitrobenzoyl chloride (1g) was dissolved in acetone (25 ml). The two solutions were mixed and allowed to stand for two hours at room temperature. The solution was then washed with ether

(50 ml, 25 ml). It was then covered with isobutyl methyl ketone (40 ml) and cooled to 0°C in an ice-bath. 2 M sulphuric acid was dropped in until no further precipitation was observed. After shaking and settling, the organic layer was separated, washed with water and dried over anhydrous sodium sulphate. To this solution was added a 50% solution of potassium 2-ethylhexanoate in butanol (2 ml). On standing overnight a pale yellow precipitate appeared. This was filtered off and washed liberally with ether. Yield 0.82g (39%). IR was identical with that of the previous sample. Mp 170-174°C. NMR: (solvent D_2O); 1.60 ppm (s, $-CH_3$); 1.70 ppm (s, $-CH_3$); 4.40 ppm (s, C_2); 5.75 ppm (s, C_5/C_6); 7.95 ppm (d, phenyl); 8.30 ppm (d, phenyl). TLC: neutral sample - 1 spot, R_f 0.68; aged in alkali - 1 spot, R_f 0.48; aged in acid - 4 spots, R_f's 0.68, 0.59, 0.48, 0.43. (Found: C, 42.7; H, 3.59; N, 10.0; S, 7.3. $C_{15}H_{14}N_3O_6SK.H_2O$ requires C, 42.75; H, 3.82; N, 9.97; S, 7.60%)

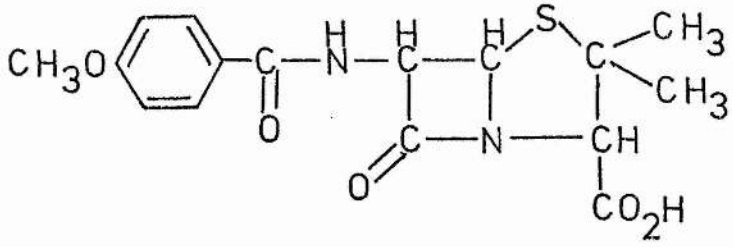
Phenylpenicillin. a) Mixed anhydride method.[49] A solution of benzoic acid (0.91g) and triethylamine (0.77g) in dioxane (16.5 ml) and acetone (3 ml) was cooled to 0°C. To this stirred solution isobutyl chloroformate (1.02g) was dropped in slowly. The rate of addition was such as to maintain the temperature below 4°C. There was a precipitation of triethylamine hydrochloride, and the mixture was stirred at room temperature for 45 minutes. A mixture of 6-APA (1.08g), triethylamine (0.5g) and water (1.5 ml) was added all at once to the mixed anhydride solution, and the mixture was stirred vigorously for one hour. A solution of sodium bicarbonate (0.55g) in ice-cold water (35 ml) was then added, and the resulting solution was extracted with ether (2 x30 ml). The aqueous solution was treated with 2M H_2SO_4 and quickly

extracted into isobutyl methyl ketone (2 x 40 ml). The combined organic layers were washed with ice-water (10 ml), dried over anhydrous sodium sulphate and filtered. A 50% solution of potassium 2-ethylhexanoate in butanol (2 ml) was added. After a few seconds, an oil began to separate. On addition of acetone (20 ml) this changed to a white solid, which was filtered off. (IR established this to be potassium benzoate.) The filtrate was set aside to evaporate, and as it did so more white powder precipitated. Yield 0.3g. IR: 3420, 1780, 1700, 1660 cm^{-1}. TLC: neutral sample - 1 spot, R_f 0.66; aged in alkali - 1 spot, R_f 0.46; aged in acid - 2 spots; R_f 0.46, 0.20. Mp 165°C. The IR suggests that the free acid has been precipitated.

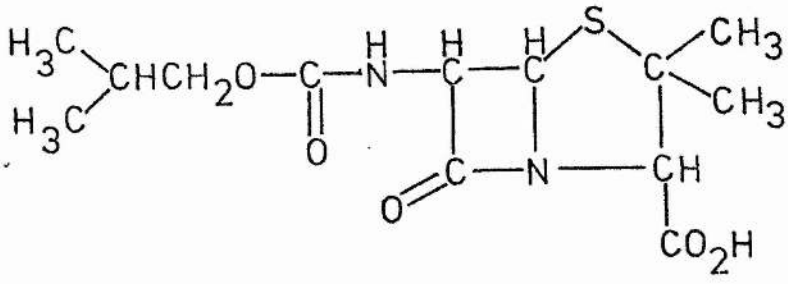
b) Acid chloride method. 6-APA (1.08g) and sodium bicarbonate (2.1g) were dissolved in water (25 ml) and acetone (2 ml), and the solution was cooled to 0°C in an ice-bath. Benzoyl chloride (0.84g) was added dropwise during one minute. This mixture was stirred vigorously for 50 minutes, while being allowed to warm up to room temperature. The mixture was washed with isobutyl methyl ketone (2 x 6 ml), and the organic extracts were discarded. The aqueous layer was covered with a further portion of isobutyl methyl ketone (19 ml), and cooled again to 0°C. 2M H_2SO_4 was added until no further precipitation occured, and the mixture was shaken so that the precipitate dissolved in the organic layer. This layer was separated, washed with cold water, dried over anhydrous sodium sulphate and filtered. A 50% solution of potassium 2-ethylhexanoate in butanol (2 ml) was added, followed by ether (50 ml). A white precipitate formed on addition of the ether. This was allowed to settle, the ether was decanted and fresh ether added. This process was repeated, and finally the mixture was filtered. (It is necessary to thoroughly purge the ketone from the precipitate, otherwise this

forms into an oil on drying.) The dried white crystalline material was added to acetone (10 ml), and the insoluble part was filtered off (potassium benzoate).. The filtrate was added to ether (50 ml), whereupon the product reprecipitated. It was washed liberally with ether as before (to wash out all the acetone) and finally filtered off and dried. Yield 0.4g (21%). Mp 175°C. IR: ν_{max} at 1760, 1650, 1600 cm^{-1}. NMR: (solvent D_2O); 1.60 ppm (s, $-CH_3$); 1.70 ppm (s, $-CH_3$); 4.35 ppm (s, C_2); 5.70 ppm (s, C_5/C_6); 7.6 ppm (m, phenyl); 7.75 ppm (m, phenyl). TLC: identical with the previous sample. (Found C, 47.6; H, 4.52; N, 7.40; S, 8.5. $C_{15}H_{15}N_2O_4SK.H_2O$ requires C, 47.86; H, 4.55; N, 7.44; S, 8.51%)

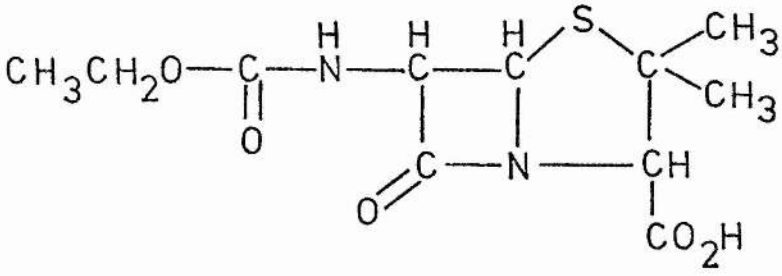
p-Methoxyphenylpenicillin. a) Attempted preparation using an isobutoxy mixed anhydride reagent. p-Methoxybenzoic acid (1.14g) and triethylamine (0.77g) were dissolved in dioxane (16.5 ml) and acetone (3 ml). This mixture was cooled to 0°C, and isobutyl chloroformate (1.02g) was added dropwise with stirring, the temperature being maintained constantly below 4°C. This mixture was stirred for 45 minutes. A cooled slurry of 6-APA (1.08g), triethylamine (0.5g) and water (1.5 ml) was added all at once, and vigorous stirring was continued for a further 2 hours. A solution of sodium bicarbonate (0.55g) in water (35 ml) was then added, and the resulting CO_2 gas was allowed to escape. The solution was washed with ether (2 x 30 ml), then acidified with 2M H_2SO_4. The precipitate was quickly extracted into isobutyl methyl ketone (2 x 20 ml). The combined organic extracts were washed with water, dried over anhydrous sodium sulphate, filtered, treated with a 50% solution of potassium 2-ethylhexanoate in butanol (2 ml) and then with ether (200 ml). The resulting precipitate was



(a.)



(b)



(c)

washed liberally with ether until it no longer became sticky on drying. Acetone (5 ml) was then added, and the insoluble portion was filtered off. The acetone solution was added to ether (100 ml), and the resulting precipitate was filtered off and dried in air. Yield 0.2g. TLC: neutral sample - 2 spots, R_f's 0.68, 0.50. IR: ν_{max} at 1760, 1710, 1650, and 1600 cm^{-1}. NMR: (solvent D_2O); 0.925 ppm (d, 9.8 cm, $-CH_3$ b); 1.65 ppm(s, 4.9 cm, $-CH_3$ a/b); 1.75 ppm (s, 4.9 cm, $-CH_3$ a/b); 3.80 ppm (t, 4.8 cm, $-OCH_3$ a, $-OCH_2-$ b); 4.25 ppm (s, 1.6 cm, C_2 a/b); 5.40 ppm (d, 0.8 cm, C_5 b); 5.60 ppm (d, 0.8 cm, C_6 b); 5.70 ppm (s, 1.6 cm, C_5/C_6 a); 7.025 ppm (d, 1.6 cm, phenyl a); 7.825 ppm (d, 1.6 cm, phenyl a). This spectrum is consistent with the sample being a mixture of p-methoxyphenylpenicillin (a) and isobutoxypenicillin (b).

b) Attempted preparation using an ethoxy mixed anhydride reagent. An identical procedure to that outlined in a) was followed; but ethyl chloroformate (0.81g) was used instead of isobutyl chloroformate. NMR: (solvent D_2O); 1.30 ppm (t, 4.5 cm, $-CH_3$ c); 1.65 ppm (s, 2.9 cm, $-CH_3$ a/c); 1.75 ppm (s, 2.9 cm, $-CH_3$ a/c); 3.80 ppm (s, 1.4 cm, $-OCH_3$ a); 4.15 ppm (q, 2.5 cm, $-OCH_2-$ c); 4.30 ppm (s, 1.0 cm, C_2 a/c); 5.4 ppm (d, 0.6 cm, C_5 c); 5.65 ppm (d, 0.6 cm, C_6 c); 5.75 ppm (s, 0.8 cm, C_5/C_6 a); 6.95 ppm (d, 0.8 cm, phenyl a); 7.80 ppm (d, 0.7 cm, phenyl a). This spectrum is consistent with the sample being a mixture of p-methoxyphenylpenicillin (a) and ethoxypenicillin (c).

c) Preparation using 6-APA silyl ester[52]. A sample of 6-APA (1.08g) was dried over phosphorous pentoxide, then suspended in dry alcohol-free chloroform (8 ml). 2 ml of chloroform which had been dried over P_2O_5 and simply decanted was added. (The P_2O_5 acts as a catalyst.) To this mixture was added hexamethyldisilazane (2 ml), care being

taken to exclude all moisture. The mixture was stirred at room temperature for one and a half hours, but this appeared to have little effect. It was gradually warmed in a water bath under reflux. When the bath temperature was 50°C, the 6-APA began to dissolve. Once it was all dissolved, 5 ml of the chloroform was distilled off, taking ammonia with it. Dry dioxane (5 ml) was added. A solution of triethylamine (0.55g) in dry chloroform (10 ml) was added, and the solution was cooled to 0°C. Anisoyl chloride (0.94g) was dropped in. The mixture was allowed to warm up to room temperature, but no precipitation occurred. (Precipitation of triethylamine hydrochloride is evidence of reaction.) The solution was heated to 50°C under reflux, then allowed to cool. It was left to stand for 16 hours. After this time, the solution had become deep red, and some solid had settled out on top. The solid was filtered off, and was identified as triethylamine hydrochloride by IR. To the red filtrate was added a mixture of ethanol (2.5 ml) and water (0.5 ml). A precipitate began to form, and after 10 minutes this was filtered off. The filtrate was treated with small amounts of a 50 % solution of potassium 2-ethyl-hexanoate in butanol, which caused the formation of an oily precipitate. After settling, the supernatant liquid was decanted, and the remaining oil was shaken with a small amount of acetone. This solution was filtered and added to several volumes of ether. The resulting pale brown precipitate was filtered off, washed with ether and dried in a dessicator over P_2O_5. Yield 0.1g (5%). TLC: neutral sample - 1 spot, R_f 0.64; aged in alkali - 1 spot, R_f 0.44; aged in acid - 2 spots, R_f's 0.64, 0.44. IR: ν_{max} at 1760, 1650, 1600 cm^{-1}. NMR: (solvent acetone-d_6); 1.575 ppm (s, $-CH_3$); 1.625 ppm (s, $-CH_3$); 3.85 ppm (s, $-OCH_3$); 4.275 ppm (s, C_2); 5.70 ppm (s, C_5/C_6); 6.975 ppm

(d, phenyl); 7.925 ppm (d, phenyl). NMR: (solvent D_2O); 1.175 ppm (s, $-CH_3$); 1.60 ppm (s, $-CH_3$); 3.475 ppm (s, C_2); 3.90 ppm (s, $-OCH_3$); 5.175 ppm (s, C_5/C_6); 7.10 ppm (d, phenyl); 7.925 ppm (d, phenyl). This spectrum is characteristic of a penicilloic acid: ie in dissolving the sample in water, the β-lactam ring is quickly hydrolysed.

Benzylpenicillin methyl ester. An ice-cold solution of penicillin potassium salt (0.6g) in water (25 ml) was acidified with 10% phosphoric acid to pH 2, and extracted with ether (3 x 20 ml). The combined ether extracts were washed with ice-cold water (30 ml) and dried over sodium sulphate in the cold. To the dried solution ethereal diazomethane[56] was added dropwise until no further evolution of nitrogen was observed. The solution was then evaporated at room temperature on a rotary evaporator to one quarter its volume, benzene (50 ml) was added, and the remaining ether was evaporated. The benzene was removed by lyophilisation, and a viscous oil was left behind. This crystallised on rubbing with hexane, and was then recrystallised from ethyl acetate/hexane. Yield 0.4g (71%). Mp 89°C (lit 90-92). IR: ν_{max} at 3400, 3380, 3340, 1790, 1770, 1755, 1740, 1690, 1675 cm^{-1}. NMR: (solvent $CDCl_3$); 1.45 ppm (d, $-CH_3$); 3.65 ppm (s, $-CH_2-$); 3.775 ppm (s, $-OCH_3$); 4.40 ppm (s, C_2); 5.575 ppm (q, C_6); 5.725 ppm (d, C_5); 6.275 ppm (d, NH), 7.35 ppm (s, phenyl).

Phenoxymethylpenicillin methyl ester. Phenoxymethylpenicillin free acid (0.5g) was dissolved in methanol (10 ml), and an ethereal solution of diazomethane was added until no further reaction was observed. The solvent was removed on a rotary evaporator at room temperature. The oily residue was taken up in methyl acetate and reprecipitated in hexane. No crystals were formed. After the oil had

settled, the solvent was decanted, the final traces being removed under reduced pressure from an oil-pump. Yield 0.45g (88%). IR: ν_{max} at 3360, 1785, 1745, 1685, 1600 cm^{-1}. NMR: (solvent $CDCl_3$); 1.55 ppm (s, $-CH_3$); 1.65 ppm (s, $-CH_3$); 3.775 ppm (s, $-OCH_3$); 4.50 ppm (s, C_2); 4.55 ppm (s, $-OCH_2-$); 5.70 ppm (m, C_5/C_6); 6.90 ppm (m, phenyl); 7.25 ppm (m, phenyl).

Phenyldiazomethane. a) By oxidation of benzaldehyde hydrazone. Hydrazine hydrate (30g, 99%) was cooled in an ice-bath, and benzaldehyde (32g) was added dropwise, the temperature being maintained below 40°C. The addition took place over 15 minutes, and the mixture was then stirred for a further 30 minutes in an ice-bath. A pale yellow precipitate was formed. This was extracted into ether (100 ml, 25 ml), washed with water (50 ml) and dried over potassium hydroxide. The ethereal solution was decanted off and concentrated in vacuo at room temperature. Yield 30g (83%). This benzaldehyde hydrazone (3g) was suspended in 40/60 petrol, and yellow mercuric oxide (5.5g) was added in small portions, the pressure being released after each addition. The reaction mixture was cooled in an ice-bath and stirred for 2 hours. The solution was then filtered, and filtered again through grade 5 filter paper. The filtrate was evaporated at room temperature, leaving a red oil which was then dissolved in ether.

b) From azibenzil. Azibenzil was prepared by the oxidation of benzil monohydrazone as follows[72,57]. Benzil (53g) was dissolved in hot ethanol (100 ml). To the solution was added dropwise, with stirring, a 60% solution of hydrazine hydrate (21g). The hydrazone began to separate from the solution after about 80% of the hydrazine had been added. The solution was refluxed for 10 minutes, then cooled in an

ice-bath. The white crystalline product was filtered off, washed liberally with ethanol, and dried in an oven below 100°C. Yield 54g (96%). Mp 146°C (lit. 149-151). This hydrazone (30g) was mixed in a mortar with yellow mercuric oxide (60g) and anhydrous sodium sulphate (15g). The mixture was placed in a 500 cm^3 glass-stoppered bottle and covered with anhydrous ether (200 ml). The mixture was cooled in an ice-bath, and a cold saturated solution of potassium hydroxide in ethanol (4 ml) was added. The mixture was shaken for 15 minutes. The resulting red solution was filtered, then filtered again through grade 5 paper, and the residue was washed several times with ether until the washings were only slightly coloured. The combined ether extracts were then filtered again through a fresh paper. The solution was evaporated to dryness at a water pump, by heating the flask to a temperature not exceeding 40°C. An orange crystalline material remained. This was made up into a saturated solution in anhydrous ether at room temperature, and placed in a deepfreeze for 3 hours. The orange crystalline precipitate was filtered off and dried in a vacuum dessicator. Yield 17g (57%). Mp 70°C (lit.74). IR: ν_{max} at 2060, 1610 cm^{-1}. To prepare phenyldiazomethane the azibenzil was further treated as follows[58]. A solution of sodium hydroxide (4g) in a mixture of water (7.5 ml) and methanol (50 ml) was added to a solution of azibenzil (2.78g) in ether (62.5 ml). The flask was loosely stoppered and allowed to stand at room temperature for 4 hours, during which time the orange colour of azibenzil changed to the red of phenyldiazomethane. At the end of the reaction the solution was filtered, then treated with 10% aqueous sodium hydroxide (50 ml). The solution separated into a red ethereal layer over a yellow aqueous layer. The ether layer was separated and washed with more of the 10% sodium hydroxide solution (4 x 12 ml), then

dried over anhydrous sodium sulphate. This ethereal solution of phenyldiazomethane may be used directly for benzylation, or it may be further purified as follows[73]. The ether is removed from the solution (but not completely, or the product is liable to spontaneously decompose) on a rotary evaporator at room temperature, and the oily product is dissolved in pentane. Contaminating by-products do not dissolve in pentane and can be filtered off. The filtrate can then be washed with water.

Estimation of phenyldiazomethane was carried out as follows. The solution would be made up to 50 ml with ether. 5 ml of this solution would be placed in a flask along with a weighed excess of p-nitrobenzoic acid. A vigorous evolution of nitrogen would ensue, with the red colour changing almost immediately to yellow. Once reaction was complete, water would be added, and a few drops of phenolphthalein. The unreacted p-nitrobenzoic acid would then be estimated by titration with 0.1M NaOH. A second 5 ml sample of the solution would be heated gently in order to decompose the phenyldiazomethane which it contained. The residue would be cooled, water added, and the same weight of p-nitrobenzoic acid as before. This solution would then be titrated against 0.1M NaOH. The difference between this titration and the previous one indicates the number of moles of phenyldiazomethane present in a 5 ml sample. Typically the yield from azibenzil is 50%.

Benzylpenicillin benzyl ester. An ice-cold solution of benzylpenicillin potassium salt (1.49g) in water (75 ml) was acidified with 10% phosphoric acid, and extracted quickly into ether (3 x 60 ml). The combined ether extracts were washed with water (100 ml) and dried over anhydrous soduim sulphate. After filtering, the solution was concentrated at the water pump to 25 ml. Phenyldiazomethane solution was added in

small amounts until no further evolution of gas was observed, and a red colour persisted in the mixture. Ether was removed from the solution at room temperature on a rotary evaporator, and a red oil was left. This was shaken vigorously with hexane; whereupon the hexane became red and a yellow oil separated out beneath it. The solvent was decanted, and the oil was washed with fresh hexane. Finally it was freeze-dried. Yield 1.2g (70%). It was not possible to crystallise this material. TLC - one spot, R_f .70. IR: ν_{max} at 3300, 1780, 1745, 1680, 1650 cm^{-1}. NMR (solvent $CDCl_3$); 1.35 ppm (s, $-CH_3$); 1.4 ppm (s, $-CH_3$); 3.55 ppm (s, $CO-CH_2-$); 4.35 ppm (s, C_2); 5.15 ppm (s, $-OCH_2$); 5.55 (m, C_5/C_6); 6.4 ppm (d, NH); 7.35 ppm (s, phenyl).

Benzylpenicillin p-nitrobenzyl ester[54]. Benzylpenicillin potassium salt (2.4g) was dissolved in water (100 ml) and cooled to 0°C. 10% phosphoric acid (10 ml) was added, and the resulting precipitate was extracted into ether (3 x 80 ml). The combined ether extracts were washed with ice-cold water (150 ml), then dried over anhydrous sodium sulphate. The ether was removed on a rotary evaporator, and the residue dissolved in dimethylformamide (30 ml). Triethylamine (0.8 ml) and p-nitrobenzyl bromide (1.26g) were added, and the solution was stirred at room temperature for two and a half hours. The solution was then poured into ice-cold water (150 ml), whereupon a white precipitate was formed. This was extracted into ethyl acetate (2 x 50 ml). The combined extracts were washed with saturated sodium bicarbonate solution (50 ml) and water (50 ml), and then dried over anhydrous sodium sulphate. The solution was concentrated by lyophilisation, and a sticky oil was left. This was dissolved in a minimal amount of ethyl acetate, and the solution was poured into ether (15 ml). A cloudy solution was formed, which was then

dropped into hexane (75 ml). A white suspension formed, which after a short time coagulated into a blob. This was removed from the solvent, and placed to dry on an evaporating dish. After a short time, the substance could be crumbled into a fine white powder. It was washed with hexane and dried in air. Yield 1.25g (42%). Mp 50°C. TLC - one spot, R_f 0.72. IR: ν_{max} at 3300, 1780. 1740. 1660, 1600 1340, 845 cm^{-1}. NMR: (solvent $CDCl_3$); 1.45 ppm (s, $-CH_3$); 1.50 ppm (s, $-CH_3$); 3.65 ppm (s, $CO-CH_2-$); 4.40 ppm (s, C_2); 5.25 ppm (s, $-OCH_2-$); 5.60 ppm (m, C_5/C_6); 6.40 ppm (d, NH); 7.25 ppm (s, phenyl); 7.50 ppm (d, p-nitrophenyl); 8.20 ppm (d, p-nitrophenyl).

Diphenyldiazomethane. Benzophenone hydrazone was prepared as follows[59]. 99% hydrazine hydrate (60g) and sodium hydroxide (60g) were placed in a 250 ml flask, connected to a condenser, with a drying tube affixed to exclude moisture. The flask was heated in an oil bath to 113°C, and the temperature was maintained within two degrees of this for two hours. It was then raised slowly to 150°C, and during this process the anhydrous hydrazine distilled over. Yield 33.5g. Bp 116°C. This anhydrous hydrazine was mixed with benzophenone (30g) and absolute ethanol (150 ml). The mixture was refluxed for 20 hours. On cooling in ice, colourless needles of the hydrazone separated. These were filtered off and dried. Yield 23.4g (72%). mp 96°C (lit. 97-98). This product (5g) and yellow mercuric oxide (5.5g) were crushed together and added to petrol 40/60 (25 ml). The flask was immersed in a water bath at room temperature, and the mixture was stirred for six hours. During this time the solution took on a deep purple colour. The mercury was filtered off from the solution, and the solvent was removed at room temperature under reduced pressure. A deep purple liquid remained which solidified on cooling.

Benzylpenicillin benhydral ester. Benzylpenicillin potassium salt (7g) was dissolved in water (30 ml). This solution was cooled in an ice-bath, acidified with 2M H_2SO_4 and quickly extracted into ether (100 ml). After washing with water and drying over anhydrous sodium sulphate, the ether was evaporated at room temperature under reduced pressure, until its volume was only 20 ml. Diphenyldiazomethane was added in small amounts to 19 ml of this, until no further evolution of gas was seen and the purple colour persisted. The remaining 1 ml of penicillin solution was then added, causing the purple colour to disappear. The ether was removed from the product under reduced pressure, and the oily residue was dissolved in methyl acetate (25 ml). This solution was shaken with 2M sodium bicarbonate solution (50 ml), separated, washed with water and dried over anhydrous sodium sulphate. On evaporation of the methyl acetate, there remained 7.2g of a pale yellow oil (74%). IR: ν_{max} at 3300, 1785, 1745, 1680, 1660 cm^{-1}. NMR: (solvent $CDCl_3$); 1.20 ppm (s, $-CH_3$); 1.40 ppm (s, $-CH_3$); 3.60 ppm (s, $CO-CH_2-$); 4.50 ppm (s, C_2); 4.65 ppm (s, -OCH-); 5.60 ppm (m, C_5/C_6); 6.475 ppm (d, NH); 7.325 ppm (s, phenyl).

6-aminopenicilloic acid. 6-APA (0.22g) was dissolved in 0.05M potassium hydroxide solution, and left to stand for sixteen hours at room temperature. During this time the pH dropped from 12.85 to 12.30. 1M hydrochloric acid was added drop by drop until the pH was 5. The solution was then lyophilised, to give a fluffy white residue. Yield 0.24g. (A mixture of the desired product and potassium chloride.) TLC: one spot - R_f .24. NMR: (solvent D_2O); 1.30 ppm (s, $-CH_3$) 1.55 ppm (s, CH_3); 3.65 ppm (s, C_2); 4.10 ppm (d, C_6); 5.2 ppm (d, C_5).

Benzylpenicilloic acid. This was prepared by the same procedure as

outlined above. TLC: one spot, R_f 0.52. NMR: (solvent D_2O); 1.25 ppm (s, $-CH_3$); 1.625 ppm (s, $-CH_3$); 3.70 ppm (s, $-CH_2-$); 3.50 ppm (s, C_2); 4.825 ppm (d, C_6); 5.35 ppm (d, C_5); 7.35 ppm (s, phenyl).

Phenylpenicilloic acid. This was also prepared by the same procedure. TLC: one spot, R_f .48. NMR: (solvent D_2O); 1.175 ppm (s, $-CH_3$); 1.60 ppm (s, $-CH_3$); 3.50 ppm (s, C_2); 5.00 ppm (d, C_6); 5.20 ppm (d, C_5); 7.575 ppm (m, phenyl); 7.825 ppm (m, phenyl).

p-nitrophenylpenicilloic acid. This also was prepared by the same procedure. TLC: one spot, R_f .46. NMR: (solvent D_2O); 1.25 ppm (s, $-CH_3$); 1.65 ppm (s, $-CH_3$); 3.55 ppm (s, C_2); 5.05 ppm (d, C_6); 5.25 ppm (d, C_5); 8.10 ppm (d, phenyl); 8.4 ppm (d, phenyl).

p-methoxyphenylpenicilloic acid. See p-methoxyphenylpenicillin for data.

Benzylpenilloic acid. Benzylpenicillin potassium salt (1g) was dissolved in water (250 ml), and 1M hydrochloric acid (3 ml) was added. This solution was refluxed for one and a half hours. Afterwards, the water was removed on a rotary evaporator. The oily residue was taken up in 10 ml of boiling water, and the solution set aside to cool. After a few hours, a mass of white crystals was deposited. Yield 0.25g (25%). mp 110°C. TLC: one spot, R_f 0.51. IR: ν_{max} at 3520, 3450, 3200, 1670, 1635, 1600 cm^{-1}. NMR: (solvent D_2O); 1.275 ppm (s, $-CH_3$); 1.625 ppm (s, $-CH_3$); 3.40 (q, $NH-CH_2-$); 3.55 ppm (s, $Ph-CH_2$); 3.625 ppm (s, C_2); 4.75 ppm (q, C_5); 7.35 ppm (s, phenyl).

Phenylpenilloic acid. Phenylpenicillin potassium salt (0.3g) was dissolved in 0.05M potassium hydroxide solution (24 ml) and allowed to stand at room temperature for three hours. 2M hydrochloric acid (2 ml) was then added, and water (100 ml). This mixture was refluxed for four hours. Water was removed on a rotary evaporator, leaving an amorphous white solid. This was washed with acetone, then taken up in a minimum amount of water. 2M hydrochloric acid was added to the concentrated solution; and the resulting precipitate was filtered off, washed and dried. Yield 0.1g (36%). mp 210°C. TLC: one spot, R_f 0.57; NMR: (solvent D_2O/K_2CO_3); 1.40 ppm (s, $-CH_3$); 1.70 ppm (s, $-CH_3$); 3.60 ppm (s, C_2); 3.80 ppm (m, $-CH_2-$); 5.00 (m, C_5); 7.50 (m, phenyl); 7.70 ppm (m, phenyl). (Found C, 48.9; H, 5.60; N, 8.10. $C_{14}H_{18}N_2O_3S.HCl$ requires C, 50.83; H, 5.79; N, 8.47%. The ratio of C:H:N is correct. The sample is contaminated with potassium chloride.)

p-nitrophenylpenilloic acid. p-nitrophenylpenicillin potassium salt (0.3g) was dissolved in water (25 ml) containing sodium hydroxide (0.1g). The resulting solution was deep orange in colour. It was allowed to stand for twenty hours at room temperature. The pH was then adjusted to 2 by dropwise addition of 1M HCl. This solution was allowed to stand at room temperature for nine days. It was then washed twice with ether and lyophilised. Yield 0.2g. TLC: one spot, R_f 0.58. NMR: (solvent D_2O); 1.30 ppm (s, $-CH_3$); 1.65 ppm (s, $-CH_3$); 3.60 ppm (s, C_2); 3.80 ppm (m, $-CH_2-$); 5.00 ppm (m, C_5); 8.10 ppm (d, phenyl); 8.40 ppm (d, phenyl).

Benzylpenillic acid[31]. Benzylpenicillin potassium salt (5g) was dissolved in distilled water (130 ml). 5M HCl was added dropwise, while the

solution was stirred. A gummy precipitate formed and collected into a lump. The flask was stoppered and left to stand at room temperature for 24 hours. During this time a mass of white crystals formed in the solution. These were filtered off, washed with water and with acetone, and dried in air. Yield 0.4g (9%). mp 180°C (lit. 194). TLC: one spot, R_f 0.36. IR: ν_{max} at 3300, 1675 cm^{-1}. NMR: (solvent D_2O/K_2CO_3); 1.50 ppm (s, $-CH_3$); 1.525 ppm (s, $-CH_3$); 3.80 ppm (s, C_2); 4.10 ppm (s, $-CH_2-$); 4.55 ppm (d, C_6); 5.65 ppm (d, C_5); 7.35 ppm (s, phenyl).

Phenylpenillic acid. Phenylpenicillin potassium salt (120 mg) was dissolved in water (27 ml), and 1M HCl solution (0.3 ml) was added. The mixture was allowed to stand at room temperature for 16 hours. After this time, sodium bicarbonate (25 mg) was added to neutralise the solution, which was then lyophilised. A white residue remained. TLC: 3 spots, R_f's 0.70, 0.45, 0.20. The residue was dissolved in a tiny volume of methanol, and the slurry was spread along 20 cm of chromatography plate covered to a thickness of 0.5 mm with silica gel. After drying, the plate was developed in a chromatank with a mixture of n-butanol, acetic acid and water (4:1:1) as solvent. The solvent was allowed to rise 14 cm above the original sample line; and this took four hours. After drying, the plate was divided horizontally into 5 mm strips; each strip was separately scraped off and collected. Each sample was then shaken with methanol (5 ml), and each solution examined by TLC. The solution which gave one spot at R_f 0.20 were pooled, filtered from the silica and evaporated at room temperature. Final drying was accomplished in a vacuum dessicator over P_2O_5. Yield 5 mg. NMR: (solvent D_2O); the spectrum was acquired over two hours, but the only discernible peaks were at 1.425 ppm (s, $-CH_3$); 1.525 ppm (s, $-CH_3$) and 7.725 (s, phenyl).

p-Nitrohippuric acid[74]. Glycine (2.51g) was dissolved in a little water, and the solution was made slightly alkaline by the addition of concentrated sodium hydroxide solution to pH 9. p-Nitrobenzoyl chloride (6.86g) was added in small amounts to this solution and stirred until it dissolved. The reaction generated acid, which was neutralised by the addition of more NaOH solution after each addition of p-nitrobenzoyl chloride. When all of the chloride had dissolved the solution was deeply red. It was mixed with its own volume of conc. HCl and its own volume of ether. The red colour was taken up by the ether layer, and a white precipitate appeared in the aqueous layer. The aqueous layer was separated and the water evaporated off. The remaining white residue was recrystallised from a small amount of hot water. Yield 1g (15%). Mp 129°C. IR: ν_{max} at 3340, 1735, 1640, 1600, 1345, 1300, 870 cm^{-1}. (Found: C, 48.1; H, 3.51; N, 12.3. $C_9H_8N_2O_5$ requires C, 48.22; H, 3.40; N, 12.50%)

2-Phenyl-4-ethoxymethylene-5-oxazolone[60]. Hippuric acid (7.2g) and triethyl orthoformate (6g) were heated for 1 hour under reflux with acetic anhydride (8g). The solution very quickly took on a deep red colour. After refluxing, low-boiling by-products were removed by heating on a steam-bath under reduced pressure. The remaining mixture solidified on cooling. Ice-cold ethanol (30 ml) was added, and the solid was stirred up with it. A pink residue remained after filtering. This was recrystallised from 80/100 petrol. Yield 3g (34%). Mp 91°C. IR: ν_{max} at 3500, 1785, 1755, 1680 cm^{-1}. NMR: (solvent $CDCl_3$): 1.45 ppm (t, $-CH_3$); 4.45 ppm (q, $-CH_2-$); 7.45 ppm (m, phenyl, vinyl); 8.10 ppm (q, phenyl). (Found: C, 66.1; H, 5.03; N, 6.51. $C_{12}H_{11}NO_3$ requires C, 66.33; H, 5.10; N, 6.47%)

2-p-Nitrophenyl-4-ethoxymethylene-5-oxazolone[61]. p-Nitrohippuric acid (3.1g) was mixed with triethyl orthoformate (6 ml), acetic anhydride (12 ml) and ethyl acetate (30 ml). This mixture was refluxed for 3 hours, during which time it became deeply red. The mixture was then kept at 5°C for 16 hours. The red precipitate was then filtered off and recrystallised twice from ethanol. Yield 1g (28%). Mp 164°C. IR: ν_{max} at 3500, 1780, 1750, 1670, 1660, 1340, 880, 860 cm^{-1}. NMR: (solvent $CDCl_3$); 1.55 ppm (t, $-CH_3$); 4.50 ppm (q, $-CH_2-$); 7.50 ppm (s, vinyl); 8.25 ppm (q, phenyl). (Found C, 54.6; H, 3.71; N, 10.6. $C_{12}H_{10}N_2O_5$ requires C, 54.94; H, 3.84; N, 10.68%)

Benzylpenicillenic acid[34]. Benzylpenicillin potassium salt (3.72g) was dissolved in water (200 ml), and this was mixed with a solution of mercuric chloride (3.26g) in water (200 ml). A precipitate formed slowly. The mixture was left to stand for 4 hours at 29°C. The penicillenic acid mercury mercaptide was filtered off, washed with water, and finally with ether. Yield 6.4g. The powder was stirred with benzene (200 ml) and water (50 ml), and cooled in an ice-bath. H_2S gas was passed in for half an hour, and a black precipitate was formed. This was filtered off. The benzene solution was washed with water, dried over anhydrous sodium sulphate and lyophilised, leaving a white powder. Yield 1.3g (39%). IR: ν_{max} at 3360, 1765, 1740, 1725, 1710, 1660, 1645 cm^{-1}. NMR: (solvent DMSO-d_6); 1.40 ppm; (s, $-CH_3$); 1.45 ppm (s, $-CH_3$); 3.85 ppm (s, $-CH_2-$); 7.30 ppm (s, phenyl, vinyl). UV: (solvent ethanol): λ_{max} at 322 nm, ε_{322} = 21,100. (Since pure benzylpenicillenic acid has ε_{322} = 26,600, this material must be 79% pure.)

Phenylpenicillenic acid. a) Via mercury mercaptide. Phenyl-

penicillin potassium salt (1.8g) was dissolved in water (100 ml). This solution was mixed with a solution of mercuric chloride (1.6g) in water (100 ml). This mixture was allowed to stand at room temperature for 24 hours, during which time a yellow precipitate formed. This was filtered off at the pump, washed with water, then dried in a dessicator over P_2O_5. Yield 1.3g. The solid was ground into a fine powder and addded to benzene (100 ml) and water (25 ml). The mixture was cooled to 8°C, and H_2S gas was bubbled in with stirring for half an hour. A black precipitate formed, and was filtered off. The yellow benzene solution was washed with water (30 ml), dried over anhydrous sodium sulphate and lyophilised. A fluffy yellow solid remained. Yield 0.15g (9%). TLC: 1 spot, R_f 0.69. IR: ν_{max} at 3340, 1725, 1640 cm^{-1}. NMR: (solvent $CDCl_3$); 1.20, 1.30, 1.45, 1.75 ppm (methyl resonances); 3.80 ppm (m); 7.40, 7.90 ppm (phenyl resonances). UV: (solvent ethanol); λ_{max} at 350 nm, $\varepsilon_{350} = 24,500$. (Found: C, 57.6; H, 5.33; N, 8.80; S, 7.1. $C_{15}H_{16}N_2O_4S$ requires C, 56.24; H, 5.03; N, 8.74; S, 10.01%)

b) Via oxazolone.[62] 2-Phenyl-4-ethoxymethylene-5-oxazolone (0.54g) and D-penicillamine (0.37g) were mixed with dry pyridine (90 ml) and triethylamine (4 ml). This mixture was heated in a water-bath at 75°C for 20 minutes. The resulting orange solution was lyophilised to a gum, which was then dissolved in chloroform (75 ml). This solution was washed with 2M phosphate buffer pH 1.6 (75 ml), then with 1.25 M phosphate buffer pH 5 (75 ml). After drying over anhydrous sodium sulphate, the chloroform solution was lyophilised. The deep red oil which remained was taken up in methyl acetate (10 ml), and the solution was poured into hexane (60 ml). A red precipitate was formed which, after filtering and drying, became orange. Yield 0.47g (59%). IR:

ν_{max} at 3300, 1730, 1640 cm^{-1}. NMR: (solvent $CDCl_3$); 1.30, 1.40, 1.50, 1.70 ppm (methyl resonances); 7.5, 7.9 ppm (phenyl resonances). UV: (solvent ethanol); λ_{max} at 353 nm, ε_{353} = 23,500. (For genuine phenylpenicillenic acid, ε_{350} = 24,300.)

p-Nitrophenylpenicillenic acid. p-Nitrophenylpenicillin potassium salt (0.8g) was dissolved in water (30 ml), and the solution was mixed with a solution of mercury chloride (0.54g) in water (30 ml). The mixture was allowed to stand for 20 hours at 5°C, during which time a red precipitate formed. This was filtered off and dried. Yield 0.7g. Over the next 5 days, more solid precipitated from the solution. When the solid was stirred with benzene (10 ml), some of it dissolved to form a yellow solution. H_2S had no effect on this solution, nor had alkaline sodium nitroprusside solution. (Thus the material is not a mercury mercaptide, nor does it possess a free -SH group. It is suggested that the mercury chloride has casued a cleavage of the penicillin molecule, and that this yellow substance is a penilloaldehyde (16)[30].) The undissolved solid was added to fresh benzene (20 ml) and water (5 ml). When H_2S gas was bubbled through this, a black precipitate formed, and the benzene layer became yellow. After filtering, the two layers were separated; the benzene was dried over anhydrous sodium sulphate and lyophilised. Yield 20 mg (5%). TLC: 1 spot, R_f 0.68. The substance gave a purple colour on treatment with alkali and sodium nitroprusside solution, indicating the presence of a free -SH group. NMR: (solvent DMSO-d_6); 1.50 ppm (d); 1.575 ppm; 3.80 ppm (m); 7.40 ppm (s); 8.25 ppm (m). UV: (solvent ethanol); λ_{max} at 400 nm, ε_{400} = 10,700. In HCl solution the maximum shifts to 378 nm, and the peak is observed to diminish with time. (Authentic

p-nitrophenylpenicillenic acid should have λ_{max} at 400 nm, with ε_{400} = 9,500. In HCl solution the maximum should be 375 nm.)

Benzyloxycarbonyl-6-APA. 6-APA (1.08g) was dissolved in water (25 ml) containing sodium bicarbonate (2.1g) and acetone (2 ml). The mixture was cooled in an ice-bath and benzyl chloroformate (0.9g) was added. This mixture was stirred vigorously for 2 hours, while being allowed to warm up to room temperature. The mixture was washed with isobutyl methyl ketone (2 x 6 ml). It was then cooled and acidified with 2M H_2SO_4. The resulting precipitate was quickly extracted into isobutyl methyl ketone (20 ml). The organic layer was washed with water, dried over anhydrous sodium sulphate and filtered. A solution of potassium 2-ethylhexanoate in butanol (2 ml) was added, followed by ether (70 ml). A white precipitate formed and was allowed to settle. The ether was decanted and fresh ether was added. Finally, the precipitate was filtered off, washed with ether, acetone, then more ether. It was dried in vacuo at 70°C. Yield 1.1g (63%). Mp 205°C. TLC: neutral sample - 1 spot, R_f 0.70; aged in alkali - 1 spot, R_f 0.54; aged in acid - 2 spots, R_f's 0.70, 0.54. IR: ν_{max} at 3470, 1770, 1725, 1600 cm^{-1}. NMR: (solvent D_2O); 1.60 ppm (s, $-CH_3$); 1.65 ppm (s, $-CH_3$); 4.30 ppm (s, C_2); 5.00 ppm (s, $-CH_2-$); 5.50 ppm (m, C_5/C_6); 7.30 ppm (s, phenyl). (Found: C, 47.2; H, 4.50; N, 6.87; S, 7.90. $C_{16}H_{17}N_2O_5SK.H_2O$ requires C, 47.28; H, 4.71; N, 6.89; S, 7.89%)

Benzyloxycarbonyl-6-APA benzyl ester. 6-APA (2.16g) and sodium bicarbonate (4.2g) were dissolved in water (50 ml) and acetone (5 ml). The mixture was cooled in an ice-bath, and benzyl chloroformate (2g) was added. The mixture was stirred vigorously for 2 hours while being

allowed to warm up to room temperature. The mixture was washed with ether (50 ml). It was then cooled, acidified with 2M H_2SO_4 and quickly extracted into ether (100 ml, 50 ml). The ether was washed with water, dried over anhydrous sodium sulphate and concentrated into 20 ml. A solution of phenyldiazomethane in hexane was added drop by drop until no further effervescence was observed and the red colour persisted in the solution. The solvent was then removed on a rotary evaporator, leaving a thick red oil. This was shaken with hexane until all of the red colour had gone into solution, leaving behind a yellow oil. This was dissolved in a small amount of ether and reprecipitated in hexane. Yield 2.5g (57%). IR: ν_{max} at 3330, 1780, 1740, 1715 cm^{-1}. NMR: (solvent $CDCl_3$); 1.40 ppm (s, $-CH_3$); 1.60 ppm (s, $-CH_3$); 4.45 ppm (s, C_2); 5.10 ppm (s, $-CH_2-$); 5.15 ppm (s, $-CH_2-$); 5.55 ppm (s, C_5/C_6); 7.35 ppm (s, phenyl).

N-benzyloxycarbonyl-N-methyl-6-APA benzyl ester.[65] N-benzyloxycarbonyl-6-APA benzyl ester (1.66g) was dissolved in dimethylformamide (14 ml). To the solution was added iodomethane (2 ml) and silver oxide (4g). This mixture was stirred at room temperature for 16 hours. At the end of this time the solid was filtered off and washed with more DMF (14 ml). To the combined filtrate was added chloroform (100 ml). A white precipitate was formed at this point (silver iodide). This was filtered off. Yield 0.9g. The chloroform solution was washed with water (4 x 50 ml) and dried over anhydrous sodium sulphate. It was then lyophilised, leaving a thick yellow oil. Yield 0.7g (41%). IR: ν_{max} at 1780, 1740, 1700 cm^{-1}. NMR: (solvent $CDCl_3$); 1.40 ppm (s, $-CH_3$); 1.625 ppm (s, $-CH_3$); 3.175 ppm (s, $N-CH_3$); 4.475 ppm (s, C_2); 5.15 ppm (s, $-CH_2-$); 5.20 ppm (s, $-CH_2-$); 5.50 ppm (s, C_5/C_6); 7.375

ppm (s, phenyl).

Methylation of phenoxymethylpenicillin. Phenoxymethylpenicillin free acid (0.35g) was mixed with DMF (7 ml), iodomethane (1 ml) and silver oxide (2g). This mixture was stirred at room temperature for 16 hours. It was then filtered, and the residue was washed with fresh DMF (8 ml). To the combined DMF filtrate chloroform (25 ml) was added, and the resulting white precipitate was filtered off. Yield 0.16g. The chloroform was removed on a rotary evaporator, and the DMF was then removed under reduced pressure from an oil-pump. A yellow oil remained. IR: 1780, 1740, 1765 cm^{-1}. NMR: (solvent $CDCl_3$); the most prominent peaks were those associated with N-methylpenicillin V methyl ester; ie 1.475 ppm (s, $-CH_3$); 1.575 ppm (s, $-CH_3$); 2.975 ppm (s, $N-CH_3$); 3.775 ppm (s, $O-CH_3$); 4.475 ppm (s, C_2); 4.625 ppm (s, $-CH_2-$, *); 5.70 ppm (q, C_5/C_6, *); 6.95 ppm (m, phenyl); 7.30 ppm (m, phenyl). Those peaks marked * were only half as intense as they should have been; and the spectrum contained many other peaks; ie 1.40 (s); 1.65 (s); 1.90 (s); 2.00 (s); 2.05 (s); 2.20 (t); 3.075 (s); 3.125 (s); 3.275 (s); 3.70 (s); 4.825 (s); 8.00 (s). It is concluded that 50 % of the product is N-methylpenicillin V methyl ester, while the other 50 % is a substance arising from attack of the methylating agent at the β-lactam nitrogen.

Attempts to remove protecting groups from N-benzyloxycarbonyl-6-APA benzyl ester. a) By hydrogenation in a basic medium.[66] N-benzyloxycarbonyl-6-APA benzyl ester (2.5g) was dissolved in methanol (100 ml) and water (25 ml). Sodium bicarbonate (0.5g) and 10% Pd/C (2.5g) was added, and the mixture was shaken in an atmosphere of hydrogen (40 psi) for 3 hours at room temperature. The equivalent of one molecule of

hydrogen was taken up. The mixture was filtered from the catalyst and lyophilised. An oily substance was left, which became crystalline on scratching with ether. Yield 0.55g (28%). TLC: 3 spots; R_f's 0.66; 0.53, 0.58. IR: ν_{max} at 1685, 1590 cm^{-1}. NMR: (solvent D_2O); 1.25 ppm (s, $-CH_3$); 1.55 ppm (s, $-CH_3$); 3.55 ppm (s, C_2); 3.70 ppm (s, $-OCH_3$); 5.10 ppm (s, $-CH_2-$); 5.15 ppm (m, C_5/C_6); 7.35 ppm (s, phenyl); 7.5 ppm (s).

b) By hydrogenation in an acidic medium[65]. Benzyloxycarbonyl-6-APA benzyl ester was mixed with methyl acetate (40 ml), methanol (40 ml), 1M HCl (5 ml) and 5% Pd/C (2.1g). The mixture was shaken with hydrogen (45 psi) for 2 hours at room temperature. The solution was then filtered from the catalyst, and triethylamine (4.3 ml) was added. The solvent was removed by lyophilisation. The residue was shaken with ether (50 ml). The solution was filtered and allowed to evaporate, leaving an oily deposit. TLC: 1 spot, R_f 0.68. NMR: (solvent $CDCl_3$); 1.10; 1.175; 1.125; 1.375*; 1.50; 1.55*; 2.90; 3.625; 3.70; 3.775; 4.45*; 5.10*; 5.15*; 5.50*; 5.825; 7.35*. This spectrum contains the peaks of the starting material (*), along with many more.

Removal of protecting group from N-benzyloxycarbonyl-6-APA.

a) Hydrogenation in a basic medium. N-benzyloxycarbonyl-6-APA was treated in the same way as its benzyl ester. The spectra of the product were identical with those of the product from the ester.

b) Hydrogenation in an acidic medium. N-benzyloxycarbonyl-6-APA was treated in the same way as its benzyl ester. The spectra of the product were identical with those obtained by hydrogenation in the basic medium.

Removal of protecting group from N-benzyloxycarbonyl-N-methyl-6-APA benzyl ester. N-benzyloxycarbonyl-N-methyl-6-APA benzyl ester (2g) was dissolved in methanol (100 ml) and water (25 ml). To the solution was added sodium bicarbonate (0.5g) and 10% Pd/C (2g). The mixture was shaken with hydrogen (40 psi) for 3 hours at room temperature. The solution was then filtered from the catalyst and immediately lyophilised. The residue was treated with 2M H_2SO_4, and quickly extracted into ether. This solution was washed with water, dried over anhydrous sodium sulphate and evaporated. IR: ν_{max} at 1780, 1750, 1700 cm^{-1}. NMR: (solvent D_2O/K_2CO_3); 1.50 ppm (s, $-CH_3$); 1.60 ppm (s, $-CH_3$); 3.025 ppm (s, $N-CH_3$); 4.35 ppm (s, C_2); 5.10 ppm (s, $-CH_2-$); 5.50 ppm (s, C_5/C_6); 7.325 ppm (s, phenyl).

Effect of trifluoroacetic acid on 6-APA. 6-APA (220 mg) was suspended in benzene (5 ml) and trifluoroacetic acid (5 ml) was added. The mixture was shaken until all of the solid dissolved, giving a yellow solution, and was then immediately lyophilised. After all of the solvent was removed, the residue was dissolved in 1% aqueous trifluoroacetic acid (5 ml). This also was lyophilised off. Finally, the residue was crystallised from methyl acetate/hexane. Yield 200 mg. TLC: 4 spots; R_f's 0.30, 0.43, 0.51, 0.60. NMR: (solvent D_2O); 1.25 ppm (s); 1.60 ppm (d); 3.475 ppm (d); 4.25 ppm (t); 4.425 ppm (d); 5.05 ppm (q).

Effect of trifluoroacetic acid on N-benzyloxycarbonyl-6-APA. Benzyloxycarbonyl-6-APA (350 mg) was suspended in benzene (5 ml) and trifluoroacetic acid (5 ml) was added. The mixture was shaken until all of the solid had dissolved, and was then immediately lyophilised.

The residue was dissolved in methyl acetate (2 ml) and the solution was added to hexane (2 ml). The resulting white precipitate was filtered off and washed with hexane. Yield 120 mg. Mp 152°C. TLC: 1 spot, R_f 0.53. IR: ν_{max} at 1730 cm^{-1}. NMR: (solvent D_2O/K_2CO_3); 1.50 ppm (s); 1.55 ppm (s); 4.25 ppm (s, C_2); 4.25 ppm (d, C_6); 5.50 ppm (d, C_5); 7.40 ppm (s, benzyl alcohol). (Found: C, 46.7; H, 4.86; N, 9.00. $C_9H_{12}N_2O_5S$ requires C, 41.53; H, 4.65; N, 10.76% $C_9H_{12}N_2O_5S.0.4C_7H_8O$ requires C, 46.96; H, 5.04; N, 9.23%) In an attempt to remove the benzyl alcohol from the sample, the product was recrystallised 5 times from chloroform. NMR indicated that no benzyl alcohol was left. (Found C, 40.2; H, 4.48; N, 10.3. $C_9H_{12}N_2O_5S.0.1CHCl_3$ requires C, 40.15; H, 4.48; N, 10.29%)

Effect of TFA on benzyloxycarbonyl-6-APA benzyl ester. A solution of the ester (220 mg) in ether (1.5 ml) was added to benzene (5 ml) and TFA (5 ml) was added. The solution initially became bright green, but after a short while it returned to yellow. The solution was lyophilised, and the resulting oily residue crystallised from methyl acetate/hexane. Yield 74 mg. Mp 92°C. TLC: 1 spot, R_f 0.65. IR: ν_{max} at 3320, 1730 cm^{-1}. NMR: (solvent $CDCl_3$); 1.35 ppm (s, $-CH_3$); 1.50 ppm (s, $-CH_3$); 4.35 ppm (s, C_6); 4.65 ppm (s, C_2); 5.20 ppm (s, $-CH_2-$); 5.80 ppm (s, C_5); 7.05 ppm (s, CO_2H); 7.40 ppm (s, phenyl); 9.45 ppm (s, NH). (Found; C, 55.7; H, 5.33; N, 7.50. $C_{16}H_{18}N_2O_5S$ requires C, 54.85; H, 5.18; N, 8.00% $C_{16}H_{18}N_2O_5S.0.12C_7H_8O$ requires C, 55.66; H, 5.25; N, 7.71%)

CHAPTER THREE

KINETIC STUDIES OF THE REACTIONS OF PENICILLINS

INTRODUCTION

The principal objective of this project has been to study the rates of reaction of penicillins with acids and bases. Effects of altering the side-chain have been investigated, along with isotope effects and esterification effects; and a picture of the overall scheme of reaction has been built up. The reactions in alkaline solution have been studied briefly, and these results are reported first in this chapter. Rather more attention has been given to reactions with dilute acid: firstly because these are more complicated; secondly because they are more relevant to the biological action of penicillin. Strongly alkaline solutions are unlikely to be encountered by the antibiotics in the body; but the stomach has a pH of ca. 2.7. For this reason it is essential that the action of acid is well understood, particularly for those penicillins designed for oral application. The second section of this chapter deals with reactions of benzylpenicillin in acid. As reported in Chapter 1, much work has been done here before. Now, old and new results have been combined to give a unified comprehensive reaction scheme. The third section deals with the reaction in acid of phenylpenicillins, and discusses the extent to which these parallel the effects discovered in the case of benzylpenicillin.

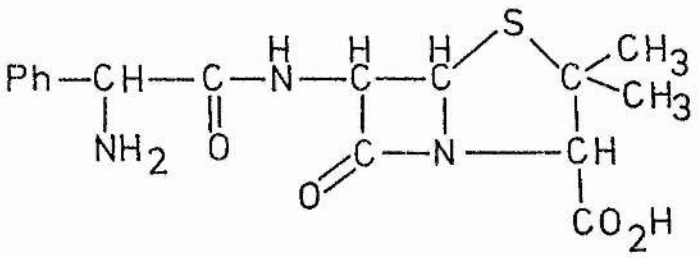
The rates of reaction have in all cases been measured by means of ultraviolet spectroscopy. The instrument used for this was a Pye-Unicam SP8-100, a highly sensitive instrument whose full scale absorbance range may be set as low as 0.05 absorbance units. The instrument is equipped with a thermostatically-controlled cell-holder

and an immersible thermocouple which gives a digital temperature readout correct to 0.1°C. As well as giving complete spectra, this instrument records optical density as a function of time, either continuously or at discreet pre-set time intervals.

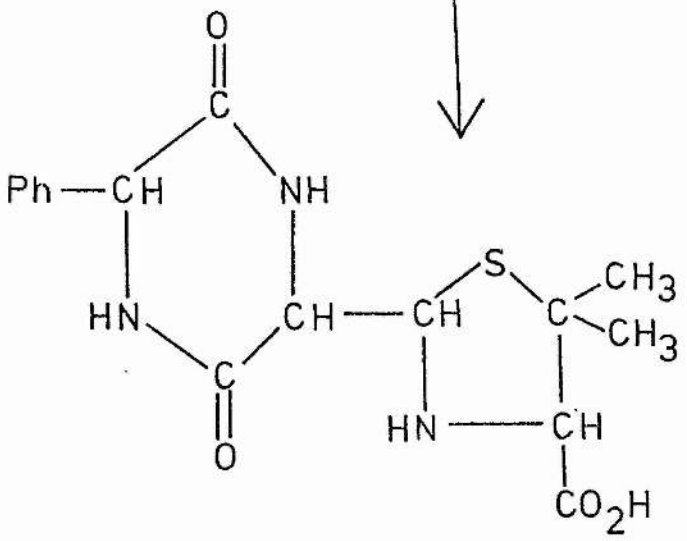
The procedure adopted for the kinetic experiments is as follows. The desired reaction medium (2.4 ml) is pipetted into a silica cuvette, and allowed to warm up to the appropriate temperature. The penicillin to be studied is prepared as a solution in either water or ethanol, with a concentration twenty-five times greater than is required for the reaction to show the optimum absorbance difference. This concentrated solution (0.1 ml) is pipetted into the pre-heated reaction medium, and the instrument is set to record the changing optical density. Conditions are chosen such that all reactions are psuedo-first-order with respect to penicillin, and the rate-constants are elucidated by processing the experimental data by the method of Swinbourne[76] (Appendix 1).

Most of the results presented here have been obtained by observing the appearance of one particular reaction product, rather than the disappearance of the penicillin itself. Despite this, and despite the fact that there is almost invariably more than one product formed, the rate constant which is calculated refers to the total reaction of the penicillin, and not just to that fraction of it which gives rise to the product under study[77] (Appendix 2).

The pH values of the buffer solution were measured on a Beckman pH meter, using a glass electrode. pD values of deuteriated buffers were measured on the same instrument, with 0.4 being added to the meter reading[78].



ampicillin



(42)

REACTIONS IN ALKALI

The action of alkali on penicillin leads to a cleavage of the β-lactam ring and formation of the penicilloic acid (25). The products of the base-inactivation of a number of penicillins have been investigated by TLC and NMR (see Chapter 2); the conclusion in almost all cases is that the penicilloic acid is the only product, and that it does not react further with the alkali. The exception to this rule was ampicillin (3e). When its reaction in alkali was examined by UV, it was found that the absorption at 230 nm at first decreased, but after a time increased again, which is not usual with penicillins. Ampicillin is known to undergo a variety of changes not characteristic of penicillins in general; eg it can polymerise[79], and can rearrange to structure (42)[80]. It is clear that the extra primary amine group causes the reaction of ampicillin to be more than usually complicated.

Neither penicillins nor penicilloic acids have particularly distinctive UV spectra; and the optical density changes caused by transforming one into the other are for the most part slight. For this reason, a study such as this one would have proved extremely difficult up until recently, before the advent of high-sensitivity spectrophotometers.

In tables 1 - 4 are listed the rate constants measured for the reactions of benzylpenicillin and phenoxymethylpenicillin at 30°C, where the ionic strength of the buffer solutions was 0.2M. Also recorded are the results for the methyl esters of these substances. For the penicillins, the decrease in absorbance at 235 nm was measured; for the esters, the increase in absorbance at 300 nm was used. All of the experiments gave good first-order plots. In figure 1, the rate constants are plotted against the concentration of hydroxide ion. For each substance, the points

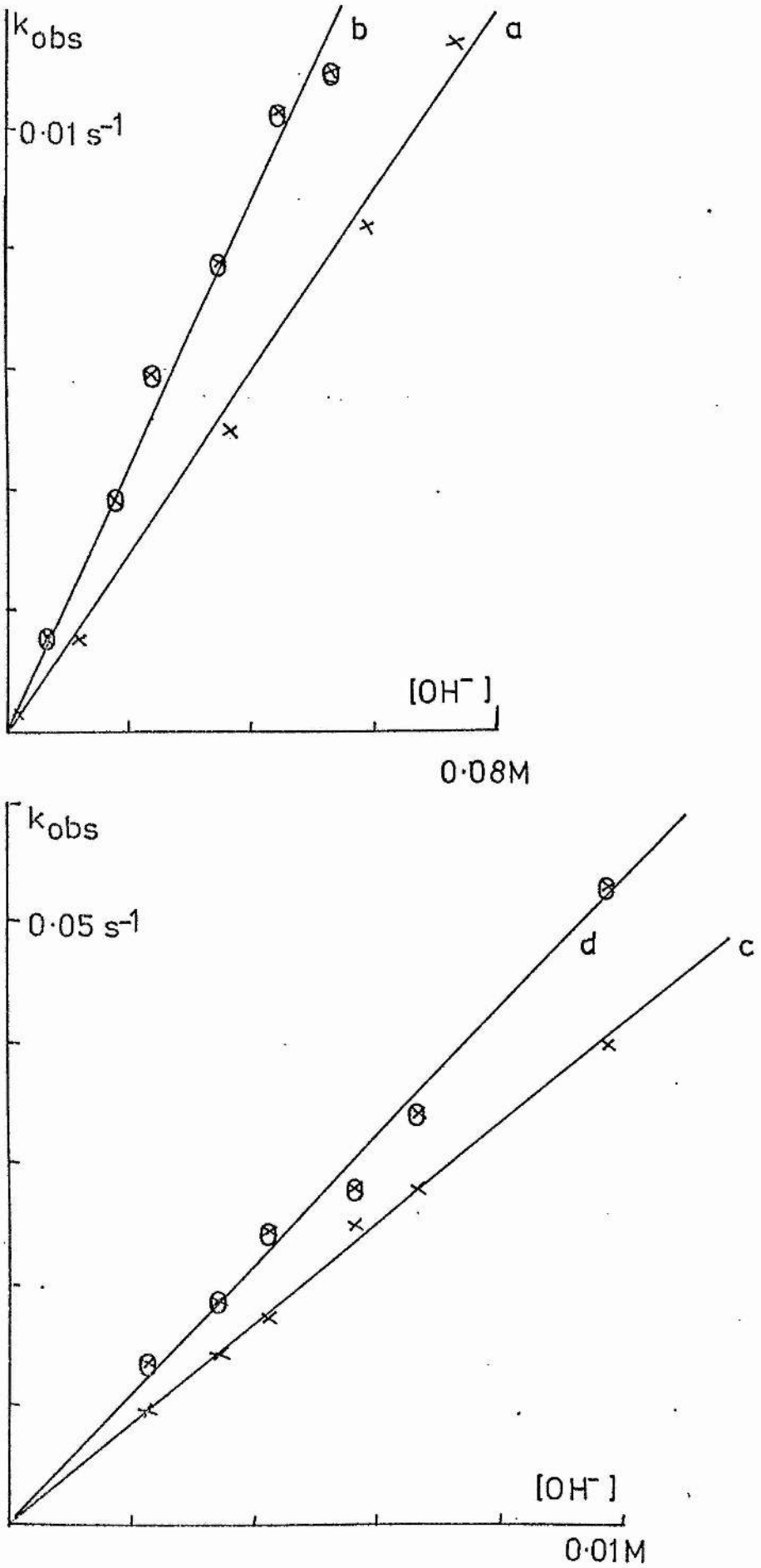


FIGURE 1. Rates of hydrolysis at 30°C. a - benzylpenicillin; b - phenoxymethylpenicillin; c - benylpenicillin methyl ester; d - phenoxymethylpenicillin methyl ester.

lie on a straight line passing through the origin. The gradient of this line can be taken as the second-order rate constant for the reaction of that substance with alkali. In all four cases the rates follow the relationship

$$k_{obs} = k_2 [OH^-]$$

The values of k_2 are summarised in table 5.

Table 1. Reaction of benzylpenicillin with alkali at 30°C.

pH	$10^5 k_{obs}/s^{-1}$	pH	$10^5 k_{obs}/s^{-1}$
12.70	1137	11.15	28.9
12.60	836	10.95	20.5
12.40	494	9.84	1.45
11.90	149		

Table 2. Reaction of phenoxymethylpenicillin with alkali at 30°C.

pH	$10^5 k_{obs}/s^{-1}$	pH	$10^5 k_{obs}/s^{-1}$
12.56	1094	12.07	379
12.49	1030	11.66	149
12.38	773	11.59	132
12.21	593	11.46	96.3

Table 3. Reaction of benzylpenicillin methyl ester with alkali at 30°C.

pH	$10^5 k_{obs}/s^{-1}$	pH	$10^5 k_{obs}/s^{-1}$
11.82	3964	11.46	1714
11.66	2779	11.38	1429
11.59	2482	11.20	951

Table 4. Reaction of phenoxymethylpenicillin methyl ester with alkali at 30°C

pH	$10^5 k_{obs}/s^{-1}$	pH	$10^5 k_{obs}/s^{-1}$
11.82	5265	11.46	2442
11.66	3393	11.38	1889
11.59	2774	11.20	1351

Table 5. Second-order rate constants for reaction with alkali at 30°C.

Substance	$k_2/M^{-1}s^{-1}$
Benzylpenicillin	0.15 ± 0.02
- methyl ester	4.05 ± 0.25
Phenoxymethylpenicillin	0.21 ± 0.03
- methyl ester	5.20 ± 0.50

The two penicillins studied here are found to have fairly similar rates of hydrolysis in alkaline solutions. This is in marked contrast to their behaviour in acidic solutions, where benzylpenicillin reacts some thirty times faster than phenoxymethylpenicillin. It is suggested that, in alkali, the reaction mechanism involves a rate-determining attack of hydroxide ion on the penicillin, as described earlier (scheme 3, page 9). Both the reactants are anions, and the intermediate therefore carries a double negative charge. Esterification removes one of these negative charges, thus making the intermediate easier to form. It is for this reason that the esters react considerably faster than the penicillins themselves. If this mechanism is correct, there is no reason why the structure of the side-chain should have a great influence on the reactivity, since it is rather remote from the reaction centre - the β-lactam ring.

For the above experiment the stock concentrated solutions of penicillin salt were made up in water, and the esters in ethanol. It was noticed that if a solution of the penicillin free acid in ethanol were used the rate constant obtained was slightly higher than for the aqueous solution. If methanol was the solvent, the rates were considerably higher. This effect is illustrated in table 6, for reactions of phenoxymethylpenicillin at 30°C.

Table 6. Effect on reaction of phenoxymethylpenicillin of varying initial solvent (4% of total).

solvent	$10^5 k_{obs}/s^{-1}$		
	pH 12.62	pH 12.25	pH 11.82
water	1352	657	214
ethanol	1582	845	309
methanol	3238	1572	877

It is difficult to account for this effect in a very satisfactory way. The concentration of methanol in the buffer is 4%, or ca. 1M, and somehow this is catalysing the reaction. Presumably it does so by lowering the free energy of the transition state relative to the reactants. It may, for example, disrupt the highly organised solvation structure of the intermediate.

REACTIONS OF BENZYLPENICILLIN WITH DILUTE ACID

A preliminary discussion of the products which arise from the

reaction of benzylpenicillin with acid has been given in Chapter 1. It is, however, important to know also the relative proportions in which these products occur; and, where intermediates are involved, the order in which they appear. This is a complex subject, because the relative proportions of each product change with the pH of the reaction medium.

It was reported earlier that when benzylpenicillin is aged in acid at pH 1, the resulting solution gives three spots on a TLC plate. This was found in three different solvent systems. One of these spots has the same R_f value as the penicillin itself; but it is unlikely that any benzylpenicillin would remain unreacted after sitting in acid for four hours. It is suggested, therefore, that the substance causing this spot is penamaldic acid (14). The R_f values of the other two spots identify these components as penicilloic acid (10) and penillic acid (12).

Dennen and Davis [43] carried out the first investigation into the relative preponderances of these latter two products throughout the acid pH range. This was done for a number of penicillins, with a variety of side-chain structures. They did, however, work under the assumption that these were the only two reaction products. Thus they measured the penicilloic acid content of the product by using a reagent specific for this (arsenomolybdic acid/$HgCl_2$), and assumed that the remainder of the product was penillic acid. They found that, for benzylpenicillin, the relative proportion of 'penillic acid' increased as the pH was increased from 1 to 3, while the proportion of penicilloic acid correspondingly decreased. It was found that 'penillic acid' was the principal product throughout this range. However, with penicillins noted particularly for their acid-resistance, such as phenoxymethylpenicillin, it was found that the proportion of penillic acid formed was considerably reduced. The authors conclude that acid-stability arises from a reduction in the rate of

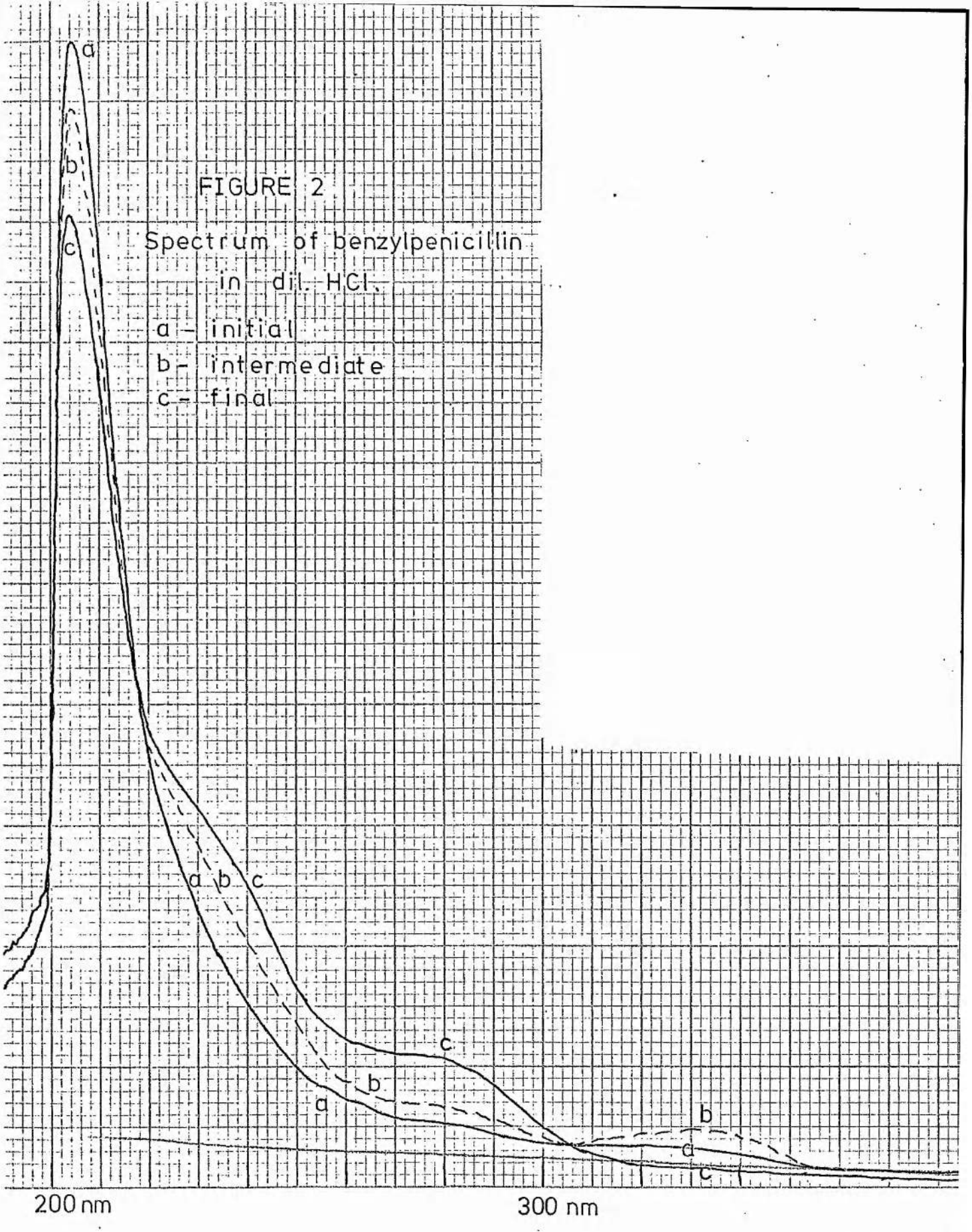


FIGURE 2

Spectrum of benzylpenicillin in dil. HCl.

a – initial

b – intermediate

c – final

formation of penillic acid; though it is evident from their results that penicilloic acid also is formed at a much slower rate from acid-stable penicillins.

These, however, are not the only products. Penicillenic acid (13) is produced; but it is an unstable intermediate, which itself reacts some ten times faster than it is formed. Its transistory existence can be demonstrated by ultraviolet studies, or by polarography. Krecji[41] first studied its formation from benzylpenicillin using the latter technique. His results indicate that the relative proportion of penicillenic acid formed increases with the pH, reaching 44% of the total at pH 2.76. From a perusal of the literature this far, it may be supposed that the 'penillic acid' spoken of by Dennen and Davis is in reality the degradation products of penicillenic acid. (Penicillenic acid itself is so reactive that it is never present in the final product.)

The reactions of benzylpenicillenic acid were considered in detail by Longridge and Timms[35]. In summary, it appears that at pH 1 the principal product is penamaldic acid, at pH 4 penillic acid is the major product, while above pH 6 penicilloic acid is formed exclusively.

Blaha et al.[32] studied the reaction of benzylpenicillin at pH 2.70, by monitoring each product by HPLC. They came to the conclusion that penicillenic acid is the only initial product, and that all other products are formed from it by subsequent reactions.

The rates of reaction of benzylpenicillin have been measured many times using a variety of techniques. Recently, a pH-rate profile was published by Page and co-workers, based on ultraviolet studies[45]. It was decided to begin this part of the project with a repeat of this work.

The ultraviolet spectrum of benzylpenicillin in an acidic buffer was observed (figure 2). The initial spectrum consisted of a single peak

with λ_{max} 204 nm. With time, the size of this peak diminished, and it took on a broad shoulder centered on 240 nm. A second peak made its appearance at 280 nm. (This is associated with benzylpenamaldic acid[81].) At 320 nm a small peak formed quickly, reached a maximum height, then slowly diminished in size. (This peak charts the formation and disappearance of benzylpenicillenic acid.) Since the reaction was known to a very complicated one, it was decided to measure the rate of change of optical density at a number of different wavelengths. The results are displayed in table 7.

Table 7. Reaction of benzylpenicillin in dilute acid at 30°C, I = 0.2M

pH	$10^5 k_{obs}/s^{-1}$			
	240 nm	280 nm	320 nm (a)	320 nm (b)
1.32	472	440	444	4530
1.38	380	403	364	3400
1.60	257	265	255	2043
1.68	219	212	210	2200
1.80	187	177	155	1800

(a) decrease of absorbance

(b) increase of absorbance

The formation of penamaldic acid, which is observed at 280 nm, is known to proceed via penicillenic acid; and the slow step is the formation of this intermediate. Therefore the rate constants calculated at this wavelength must refer to the total reaction of the penicillin (Appendix 2). They are in good agreement with the rate constants measured at 240 nm.

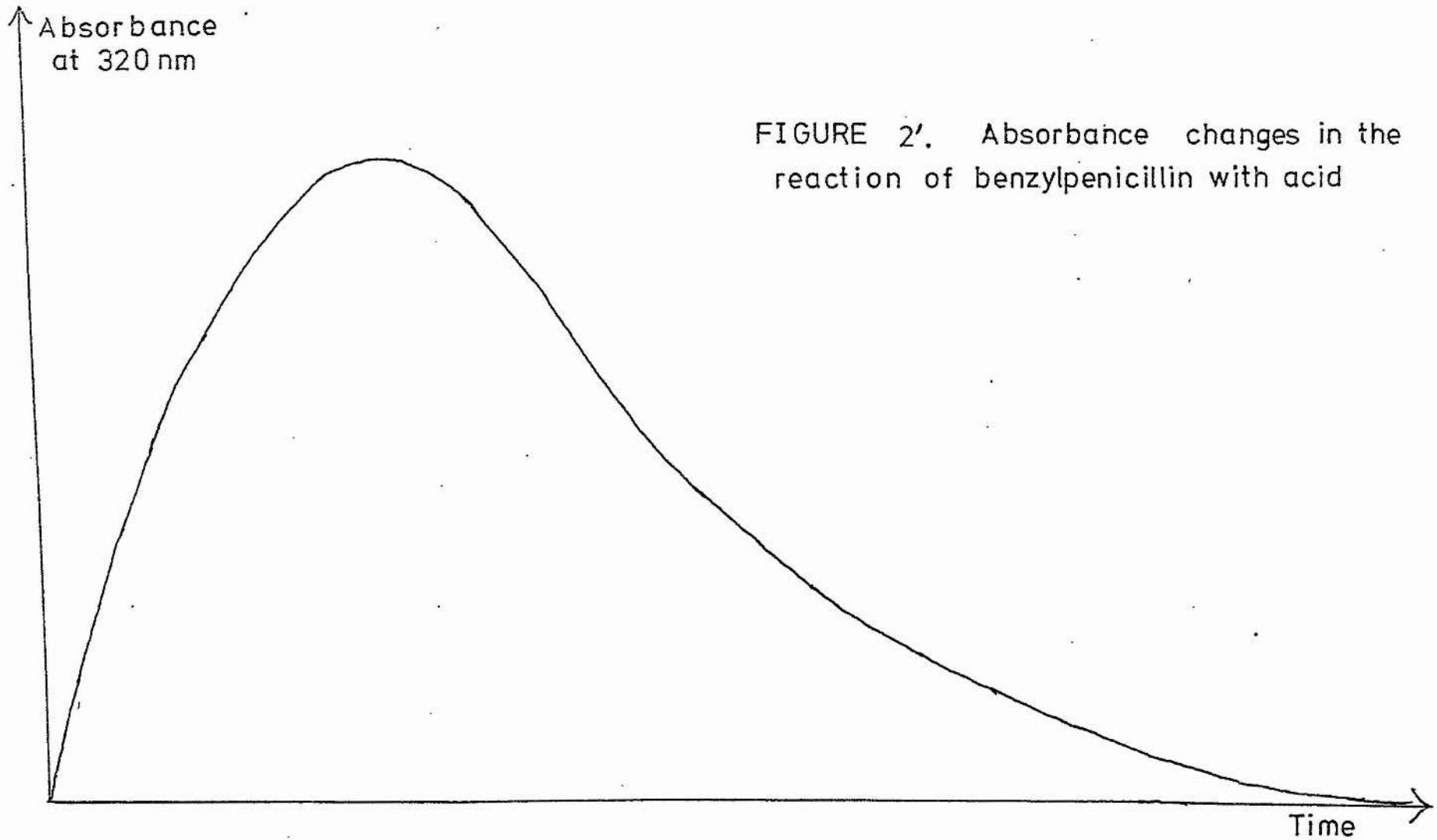


FIGURE 2'. Absorbance changes in the reaction of benzylpenicillin with acid

The situation at 320 nm is more complicated; two consecutive reactions each bring about changes in the optical density. It is possible to analyse both parts of the absorbance/time curve (figure 2') by the Swinbourne method and thus obtain rate constants. It can be shown (Appendix 3) that, to a rough approximation, the rising part of the curve yields the rate constant for the faster of the two reactions, while the falling part yields the slower rate constant.

In order to test how good these approximations could be expected to be, a mathematical model of the consecutive reactions problem was set up on a computer. The computer was programmed to print the graph of [B] vs time for the reaction sequence

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

Graphs were produced for a variety of values of k_1 and k_2. Each resembled figure 2' and was analysed by the Swinbourne method, to yield two 'apparent' rate constants k_1' and k_2'. It was found that, if k_1 and k_2 were very close in value, the 'apparent' rate constants were greatly different from the real ones. The analysis always gave at least a fourfold difference in value between k_1' and k_2'. However, if k_1 and k_2 were more than fourfold different, k_1' and k_2' gave reasonable approximations to them. The slower constant in fact would be almost indistinguishable from the real one, while the faster one would be about 20% higher than the real one.

In considering the benzylpenicillin case, it is apparent that the two sets of rate constants measured at 320 nm are sufficiently different. The lower set therefore corresponds to the disappearance of the penicillin; and these are in good agreement with the values obtained at other wavelengths.

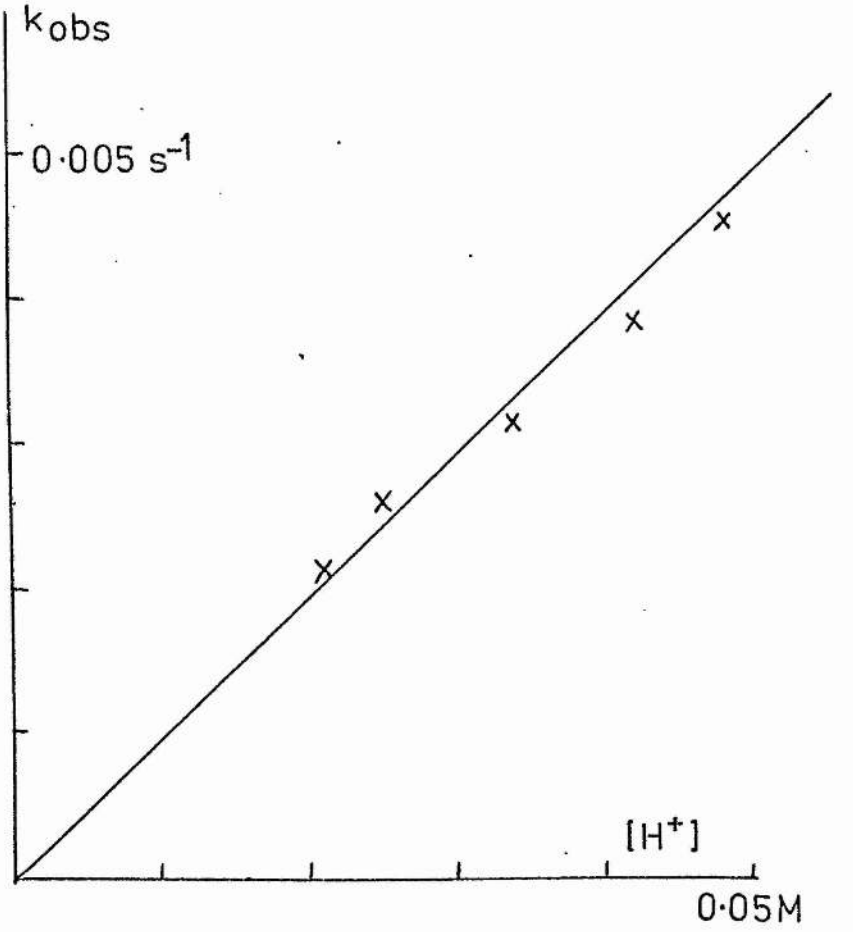


FIGURE 3. Reaction of benzylpenicillin with acid at 30°C.

These have been averaged and are presented in table 8.

Table 8. Reaction of benzylpenicillin in dilute acid at 30°C.

pH	$10^5 k_{obs}/s^{-1}$	pH	$10^5 k_{obs}/s^{-1}$
1.32	452	1.80	173
1.38	382	1.95	128
1.47	314	2.97	24.6
1.60	259	3.50	10.6
1.68	214		

If k_{obs} (for pH < 1.8) is plotted against $[H^+]$, a straight line is obtained (figure 3) whose gradient is 0.095 ± 0.008 $M^{-1} s^{-1}$. This is in good agreement with the second-order rate constant reported by Brodersen[39] (0.092 $M^{-1}s^{-1}$). It relates to the inactivation of the free acid form of the penicillin. The corresponding rate constant for the penicillin anion was reported by Brodersen as 0.375 $M^{-1}s^{-1}$.

The faster set of rate constant measured at 320 nm should correspond to the reaction of penicillenic acid. To test this, some authentic benzylpenicillenic acid was obtained (from Sigma Company), and the rate constants for its reaction with dilute acid were measured by following the decrease of absorbance at 320 nm. In the same buffer solutions, benzylpenicillin was reacted, and the increase of absorbance at 320 nm was followed. The results are summarised in table 9.

Table 9. Reaction of benzylpenicillenic acid in dilute acid at 30°C.

pH	$10^5 k_{obs}/s^{-1}$	
	from benzylpenicillin	from benzylpenicillenic acid
1.26	4900	4200
1.45	3000	2600
1.70	1900	1600
1.94	1100	1000

In each case, the rate measured in the penicillin experiment is some 10 - 20% higher than the authentic value. From the nature of the approximation, this is what would have been expected; and it is good evidence that the intermediate causing the absorbance at 320 nm is benzylpenicillenic acid. The second-order rate constant for the reaction of benzylpenicillenic acid with acid (pH $<$ 2) is therefore $0.80 \pm 0.07\ M^{-1} s^{-1}$.

Penicillenic acid is an intermediate which is formed throughout the acid pH range. A set of experiments was performed with the object of determining how much of it is produced at various pH values. The method chosen was to compare the maximum optical density at 320 nm recorded during an experiment with a theoretical maximum calculated on the assumption that all the penicillin is transformed to penicillenic acid. Dividing the actual value by the theoretical value gives the percentage of pencillenic acid formed at that pH.

It can be shown (Appendix 3) that the theoretical maximum (OD_{max}) is a function of the initial concentration of penicillin in the buffer (A_o), the extinction coefficient of penicillenic acid at that pH (ε_{320}) and the rate constants for reaction of penicillin (k_1) and penicillenic

Table 10: Calculation of percentage of penicillenic acid formed from benzylpenicillin at 30°C.

pH	$10^4 A_o/M$	10^{-4} $_{320}$	$10^5 k_1/s^{-1}$	$10^5 k_2/s^{-1}$	t_{max}/s		$O D_{max}$		% of
					calc	obs	calc	obs	penicillenic acid
1.28	2.25	2.82	452	4000	6	60	0.541	0.048	8.8
1.47	2.25	2.51	314	2480	95	130	0.530	0.059	11.1
1.95	2.25	2.32	128	1050	229	242	0.476	0.106	22.3
2.93	1.25	2.12	27.6	508	606	617	0.121	0.081	66.9

acid (k_2) at that pH. The time taken to reach this maximum (t_{max}) is a function of these four parameters, and it should be independent of the actual percentage conversion to penicillenic acid.

For each of the buffer solutions to be considered, k_1 and k_2 were measured in the usual way. ε_{320} was measured by placing an exactly known concentration (C) of penicillenic acid in the buffer and recording the optical density (OD) at a fixed time (t) after the beginning of the reaction. The extinction coefficient is then given by

$$\varepsilon_{320} = OD/Ce^{-k_2 t}.$$

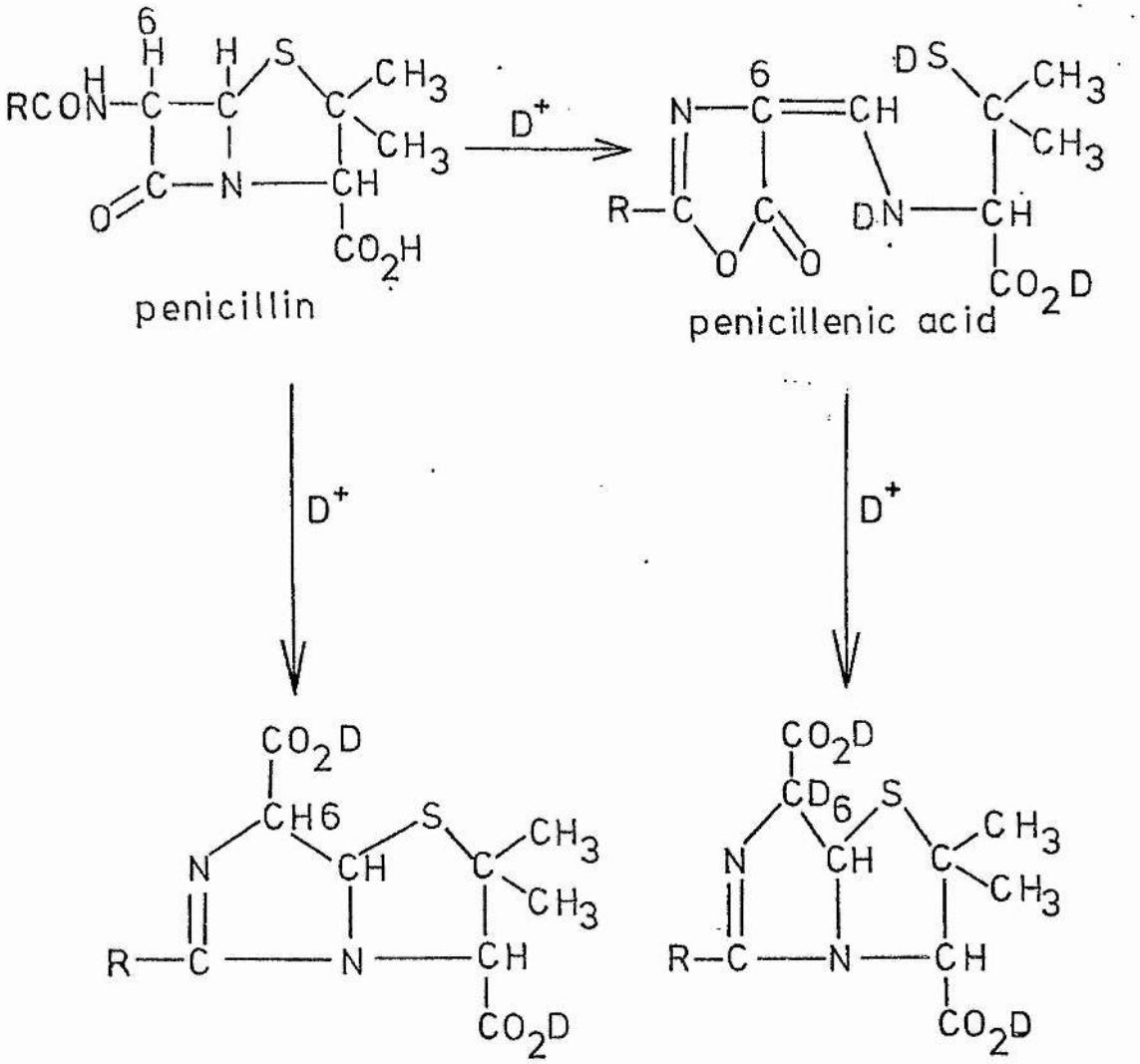
For a given A_o, t_{max} and OD_{max} were calculated. They were then determined experimentally. The results are given in table 10.

It can be seen that the percentage of penicillenic acid rises continuously with the pH. These results are broadly similar to those obtained by Krecji at 25°C by the polarographic method.

What of the remainder of the product which is not penicillenic acid? A considerable proportion of this must be penicilloic acid. This is a major product of pencillin degradation, but it is not formed from penicillenic acid at pH < 4[35]. It must therefore be formed directly from the penicillin. Dennen and Davis[43] have reported the relative amounts of this product formed. From their findings, table 11 can be drawn up.

Table 11. Proportion of benzylpenicilloic acid formed at 35°C.

pH	1.28	1.47	1.95	2.93
% of penicilloic acid	45	43	37	13



SCHEME 21

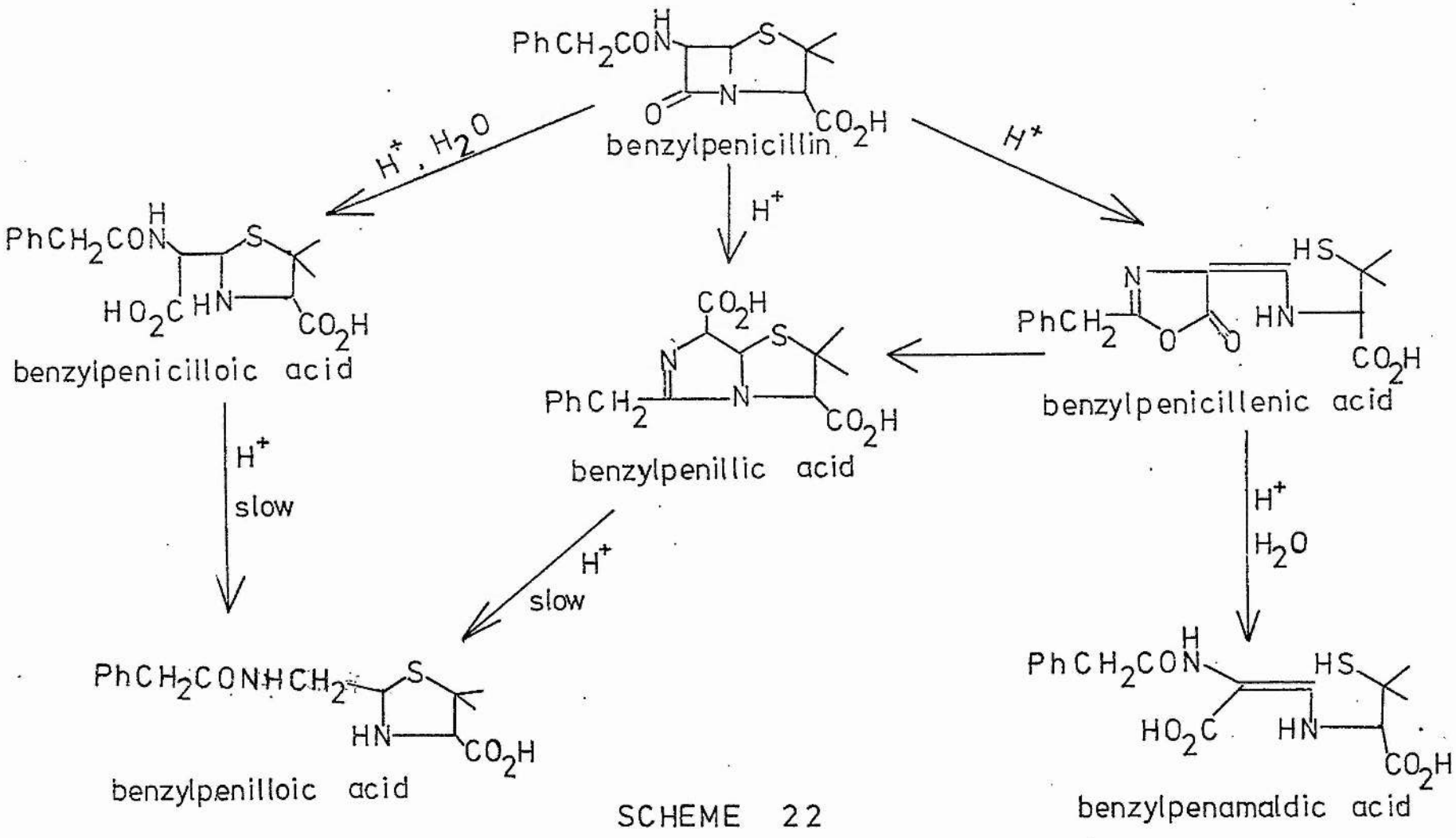
It is apparent that, especially at the low pH values, these two products do not account for all of the penicillin which has reacted. There must be a third primary degradation product. The most likely candidate for this is penillic acid. This is known to be formed from penicillenic acid, but not in very substantial amounts at pH 1. Since it is a major degradation product of penicillin at pH 1 (evidence from the chromatography studies) it must be a primary as well as a secondary product. The amount of penicillin which is initially converted to penillic acid may be estimated by subtraction of the percentages of the other two primary products from 100. Table 12 may then be drawn up.

Table 12. Proportion of penillic acid formed from benzylpenicillin.

pH	1.28	1.47	1.95	2.93
% of penillic acid	46	46	41	20

Evidence that penillic acid is formed by two different routes has been reported by Feeney et al.[44]. In a study of the inactivation of benzylpenicillin at pH 2.5 and 37°C by Fourier Transform NMR spectroscopy, they were able to distinguish between penillic acid formed by each of the two routes. If it is formed directly from the penicillin the proton will remain at position 6. If it is formed via penicillenic acid, however, this proton must be replaced by a deuteron (scheme 21). The NMR spectrum will therefore be different for each. The authors have estimated the relative amounts of each primary product formed at this pH. Their results are included in table 13, which summarises what has gone before.

It must be emphasised that these are only the initial products.


benzylpenicillin
H+, H2O
H+
H+
benzylpenicilloic acid
benzylpenillic acid
benzylpenicillenic acid
H+
slow
H+
slow
H+
H2O
benzylpenilloic acid
benzylpenamaldic acid

SCHEME 22

Table 13. Percentages of primary degradation products from benzylpenicillin.

pH	Penicilloic acid	Penillic acid	Penicillenic acid
1.28	45	46	9
1.47	43	46	11
1.95	37	41	22
2.50	17	33	50
2.93	13	20	67

Each one itself undergoes changes, some slow some fast, into other products. A more complete scheme is given in scheme 22.

The rate of hydrolysis of benzylpenicillin in deuteriated buffers was measured. The buffers were prepared by mixing 20% DCl/D_2O with D_2O. The results are displayed in table 14, along with the values expected for the rate constants in ordinary buffers.

Table 14. Reaction of benzylpenicillin in D_2O buffers at $30^{\circ}C$.

pD	$10^5 k_{D_2O}$ /s^{-1}	$10^5 k_{H_2O}$ /s^{-1}	k_{D_2O} /k_{H_2O}
1.06	2240	825	2.71
1.31	1020	470	2.17
1.52	654	287	2.27
2.58	70.3	57	1.23

This exercise was also carried out for benzylpenicillenic acid. The results are displayed in table 15.

The significance of these findings will be discussed later in the chapter.

Table 15. Reaction of benzylpenicillenic acid in D_2O buffers at 30°C

pD	$10^5 k_{D_2O}$ /s^{-1}	$10^5 k_{H_2O}$ /s^{-1}	k_{D_2O}/k_{H_2O}
1.06	16500	6950	2.37
1.31	8130	3900	2.08
1.65	3570	1800	1.98
2.52	458	250	1.83

REACTIONS OF THE PHENYLPENICILLINS WITH DILUTE ACID

The reactions of phenylpenicillin, p-nitrophenylpenicillin, p-methoxyphenylpenicillin and methicillin were examined by ultraviolet spectroscopy. The spectra of all four consist initially of single peaks, located between 200 and 300 nm. On taking the spectra in 0.1M HCl, the size of these peaks is observed to increase with time. Also, a second peak makes its appearance above 300 nm. With time, this peak reaches a maximum and slowly diminishes again. The wavelengths of these peaks are reported in table 16. In the case of methicillin, a third peak appeared in the spectrum at 280 nm.

Table 16. Absorption maxima for penicillins in 0.1M HCl.

Penicillin	Original peak	Intermediate peak
phenyl-	250 nm	350 nm
p-nitrophenyl-	267 nm	375 nm
p-methoxyphenyl	255 nm	360 nm
methicillin	210 nm	330 nm

The increase-decrease effects which are observed above 300 nm may be attributed to the formation and decay of the penicillenic acids, analogous to the benzylpenicillin case. The λ_{max} value is higher for the phenylpenicillenic acids than for benzylpenicillenic acid, because there is extra conjugation between the benzene and oxazolone rings.

Although penicillenic acids react in dilute acid solution, this reaction may be stopped by formation of a mercuric mercaptide complex[82]. Thus when the penicillins were aged in acidic solutions containing equivalent amounts of mercuric chloride, these penicillenic acid peaks were observed to remain in the spectra for at least 24 hours.

Table 17 records the maximum absorbances which the penicillins achieve in 0.1M HCl solution, both with and without the mercuric chloride. The initial concentration of penicillin is 10^{-4}M.

Table 17. Maximum optical densities of 10^{-4}M solutions at pH 1.

Penicillin	λ / nm	A_{max}	A_{max} ($HgCl_2$)
benzyl-	320	0.06	0.30
phenyl-	350	1.31	1.58
p-nitrophenyl-	370	1.00	0.81
methicillin	330	0.48	0.92

Since the extinction coefficients for phenyl- and benzyl-penicillenic acids are roughly similar (24,300; 26,600), it may be concluded that far more of the former than the latter is produced at pH 1. Also, the maximum level of phenylpenicillenic acid achieved in solution is not greatly increased by the addition of mercuric chloride,

whereas that of the benzylpenicillenic acid is increased fivefold. This indicates that phenylpenicillenic acid is relatively stable in acid solution, and decays rather more slowly than it is formed. This is the reverse of the situation with benzylpenicillin. p-Nitrophenylpenicillin appears to behave in a manner similar to phenylpenicillin, while methicillin represents an intermediate case.

Attempts at the preparation of penicillenic acids were described in the last chapter. Although the preparations were not pure, their UV spectra had the maxima noted above. This provides further evidence of the formation of penicillenic acids as intermediates in the acid-degradation processes of all these penicillins.

To date, further work on product analysis has been carried out only in the case of phenylpenicillin. This has been investigated by TLC and NMR.

TLC EXPERIMENTS

Phenylpenicillin potassium salt (40 mg) was dissolved in water (0.1 ml) and 0.01M HCl (10 ml) was added. At 4 minute intervals 1 ml portions of this solution were neutralised with 0.05M K_2CO_3 solution (0.1 ml). These neutralised solutions were examined by TLC. All ten chromatographs had three spots with R_f values 0.70, 0.45, and 0.20. The first few had the 0.70 spot as the predominant one, but after that the other two spots became of similar importance. The experiment was repeated, using 0.1M HCl and 0.5M K_2CO_3. The same spots were observed, but after 40 minutes the 0.70 spot had completely disappeared.

The 0.70 spot may correspond either to the penicillin or the penicillenic acid, both of which disappear with time. It would have been expected (from analogy with benzylpenicillin) that phenylpenamaldic

acid also would have an R_f value around 0.70. These results, then, may indicate that this is not a significant product. The 0.45 spot corresponds to phenylpenicilloic acid. In this timescale it does not undergo decarboxylation to penilloic acid (R_f = 0.57), but this change was observed if the solution was left to stand for about a week. The 0.20 spot most probably corresponds to phenylpenillic acid.

NMR EXPERIMENTS

It was established by trial and error that the greatest concentration of phenylpenicillin which could be obtained in a solution of pH 2 was about 4 mg/ml (ca. 0.01M). A solution of this concentration in D_2O gave a clear, sharp NMR spectrum after 2000 scans (ca. 2 hours).

KD_2PO_4 was prepared by dissolving KH_2PO_4 in D_2O and allowing the solvent to evaporate. This was repeated, and the remaining moisture was driven off by heating in an oven.

A deuteriated buffer solution (0.2M, pD = 2.26) was prepared by dissolving KD_2PO_4 (0.552g) and DCl (1.183 moles) in D_2O (20 ml). Neutralisation of this required 0.0207g of K_2CO_3 for every millilitre of solution to be neutralised.

A solution of phenylpenicillin potassium salt (4 mg/ml) in the solution gave an NMR spectrum not quite so good as the first one, but still clearly recognisable (figure 4a).

As a preliminary, the reaction was studied by UV spectroscopy. The deuteriated buffer solution was preheated in the spectrometer to 30°C, and phenylpenicillin was introduced to a concentration of 0.04 mg/ml (ie 100 times more dilute than for the NMR experiments). The optical density reached a maximum of 1.55 units after 12 minutes.

To study the degradation of phenylpenicillin by NMR the following procedure was adopted. The buffer solution was maintained at 30°C in a water-bath. Phenylpenicillin potassium salt (2.4 mg) was weighed out into a test-tube, also maintained at 30°C. The buffer solution (0.6 ml) was pipetted onto the penicillin, and the mixture was shaken to dissolve. The test-tube was kept in the water-bath for a measured length of time; then the solution was neutralised by addition of K_2CO_3 (0.0124g) and TMPSA (0.6 mg). The acquisition of the NMR spectrum was begun within 15 minutes and lasted for about 2 hours.

In the NMR spectrum, the most interesting region is 1-2 ppm. This is where the $-CH_3$ protons resonate. Since there are six of these on the molecule, they give rise to the most intense peaks; and the positions of these peaks are sufficiently different in the various reaction products for an analysis of them to be diagnostic. The C_5/C_6 resonances would be expected to provide more interesting information, but they are not so intense and they tend to occur too close to the H_2O peak. The spectra obtained in these experiments between 1 and 2 ppm are displayed in figure 4. A summary of the information available from them is given in table 18.

It seemed surprising that, after 20 minutes, phenylpenicillin should still be the most significant component of the mixture. Not only in the methyl region, but throughout the spectrum, could its peaks be clearly identified.

In order to check that the reaction was proceeding in the same way as determined by UV on the more dilute solution, a sample was aged in the buffer in the standard manner for 12 minutes. It was then neutralised , diluted one hundred fold with water and examined

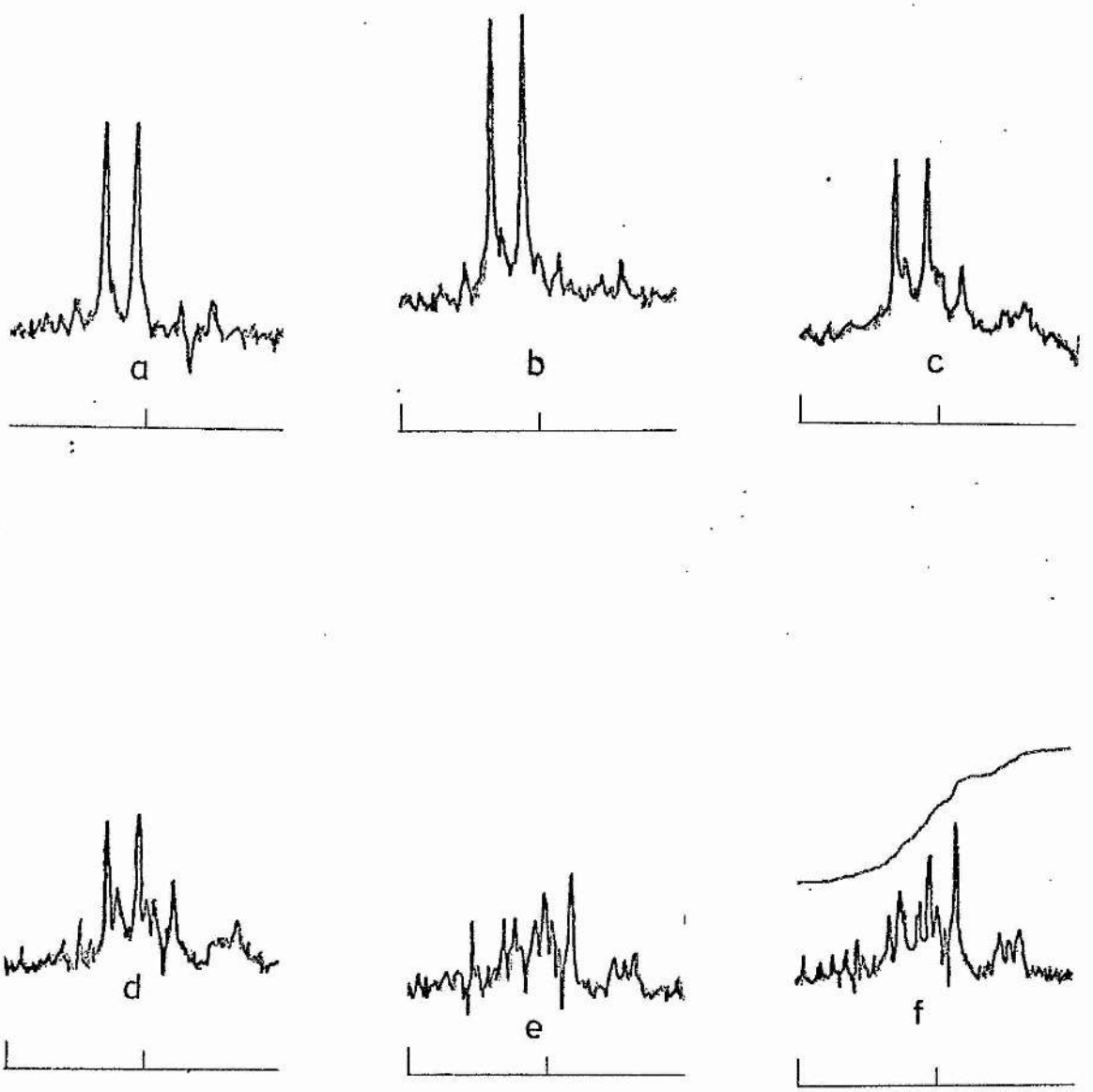


FIGURE 4

Reaction of phenylpenicillin with acid

NMR spectra: 1—2 ppm

by UV. λ_{max} was at 350 nm, with OD = 1.54 units. Thus the two sets of experiments do seem to take the same course. However, if the neutralised solution were left to stand for 2 hours, then diluted by 100, the optical density at 350 nm was only 0.38. It may be concluded that the penicillenic acid itself degrades continuously during the time necessary to acquire the NMR spectrum, even in the neutral solution. It therefore does not receive enough scans for its spectrum to be differentiated from the noise; neither do the products of its degradation. Thus, the penicillin is the only species which can give rise to a 'well-resolved' spectrum, even though (from the ultraviolet evidence) half of it has reacted after 12 minutes.

Table 18. Reaction of phenylpenicillin at pH 2.26 at 30°C.
List of methyl resonances, in order of intensity.

Spectrum	time /min	Peaks
a	0	1.55, 1.65
b	4	1.55, 1.65 1.20, 1.425, 1.625, 1.75
c	12	1.55, 1.65 1.225, 1.425, 1.50, 1.625, 1.75
d	20	1.55, 1.65 1.225, 1.425, 1.50, 1.625, 1.75
e	60	1.40, 1.50 1.55, 1.60, 1.65, 1.70 1.225
f	90	1.40, 1.50 1.625 1.55, 1.65 1.225
g	12	1.55, 1.65 1.60, 1.475, 1.40, 1.225, 1.175

Spectrum g) was obtained when the sample was allowed to react in the usual way for 12 minutes. It was then neutralised and left to stand for 2 hours before the NMR acquisition was begun. As a result, the product peaks are more intense than in spectrum c).

The peaks which appear at 1.625 and 1.225 ppm may be assigned to phenylpenicilloic acid. The reason why the lower of these resonances is split may be that acid hydrolysis leads to a mixture of isomers (5R,6R and 5S,6R) whereas alkaline hydrolysis (by which the standard phenylpenicilloic acid was obtained) gives only the 5R,6R isomer. Similar results are obtained with benzylpenicillin[44].

The peaks at 1.40 and 1.50 ppm are most probably due to phenylpenillic acid. It has already been established that the product with R_f = 0.20 has these peaks, and this is thought to be the penillic acid.

There are other peaks not accounted for, but they do not appear consistently in all the spectra. It may be that they are spikes.

In summary, the evidence gathered so far is consistent with the view that the reactions of phenylpenicillin follow the same broad pattern as those of benzylpenicillin. The striking difference is that the penicillenic acid is formed in much greater abundance, and reacts much more slowly with the acidic buffer. At low pH the major product from benzylpenicillenic acid is the penamaldic acid. In the phenyl case, there is no evidence that a penamaldic acid is formed at all. We cannot say with certainty that it is not formed, but it would be expected to have a high R_f value and to possess a distinctive UV spectrum.

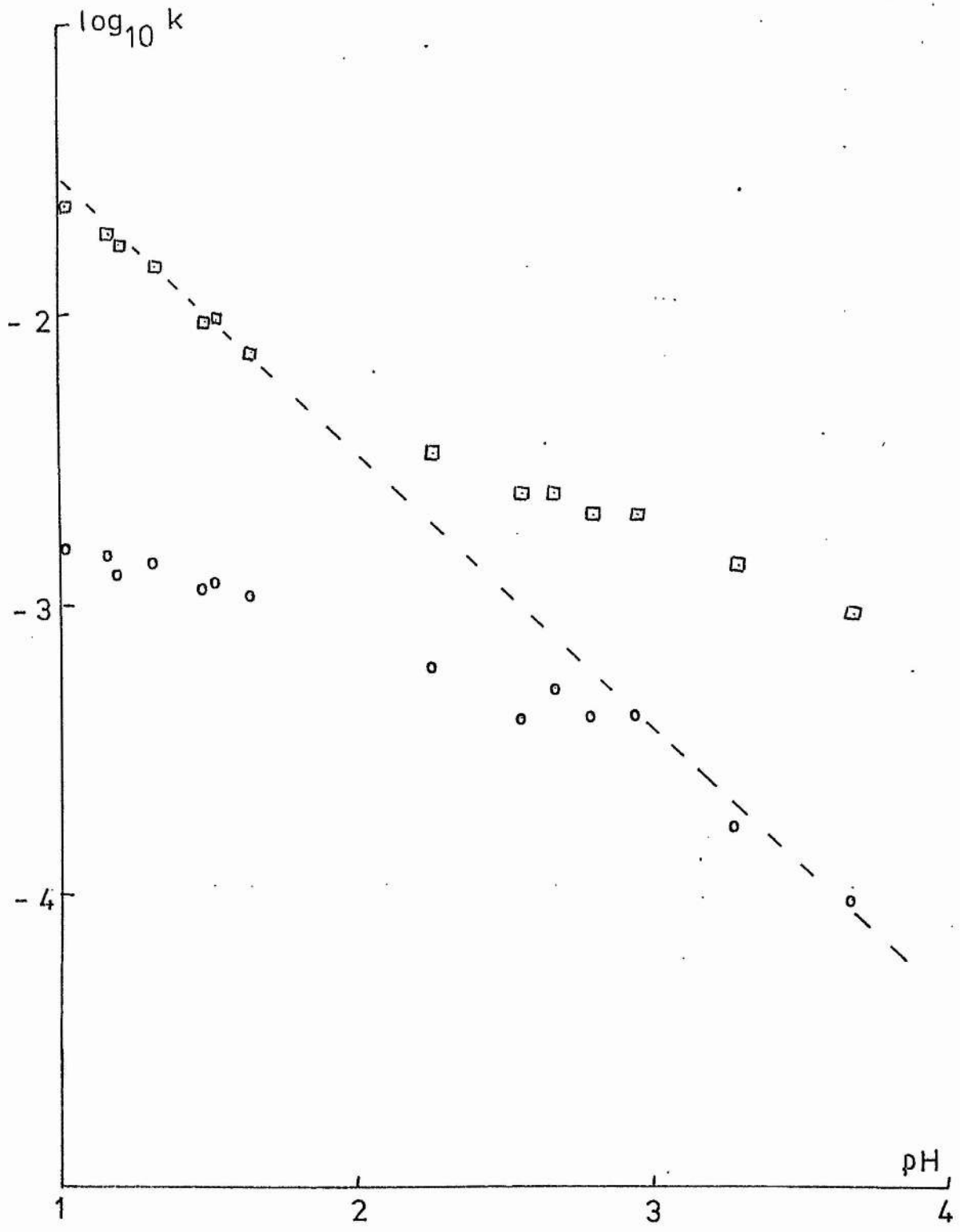


FIGURE 5: Rates of change of absorbance (350 nm) in reaction of phenylpenicillin with acid.
□ - k_1 ; o - k_2

KINETIC EXPERIMENTS

A. PHENYLPENICILLIN.

Table 19 lists the rate constants for the increase (k_1) and decrease (k_2) of absorbance at 350 nm for solutions of phenylpenicillin at 30°C. Also recorded are the maximum absorbance values (A_{max}) obtained for each solution at this wavelength. The rate constants have been calculated by the Swinbourne method. They are approximations, subject to the limitations discussed on page 62.

Table 19. Reaction of phenylpenicillin (0.02g l^{-1}) at 30°C.

pH	$10^5 k_1$ / s^{-1}	$10^5 k_2$ / s^{-1}	A_{max}
1.02	2400	160	1.00
1.15	1900	150	1.10
1.17	1750	130	1.15
1.30	1500	140	1.15
1.47	960	115	0.80
1.51	990	120	0.78
1.64	760	110	0.77
2.27	340	62	0.63
2.56	250	40.5	0.56
2.67	250	52	0.53
2.79	205	42	0.49
2.94	210	42.5	0.35
3.28	140	18	0.29
3.66	97	9.9	0.23

These results are displayed graphically in figure 5. As the pH is increased there is a steady decrease in the maximum absorbance

achieved during the experiment. This can be attributed to a relative decrease in the stability of the penicillenic acid as the pH is raised. Thus at low pH, the formation of this intermediate occurs much faster than its decay; in the pH range 3-4 the reverse is true. At intermediate values, the two processes take place at roughly similar rates. For pH < 2, then, k_1 should represent a good approximation to the rate of reaction of phenylpenicillin, while k_2 represents the rate of reaction of phenylpenicillenic acid. In the range 3-4, k_2 represents the reaction of the penicillin and k_1 that of penicillenic acid. In the range 2-3, the measured constants are too close together to have any real meaning.

The experiments were repeated, but this time mercuric chloride was added to the buffers, to a concentration of 10^{-4}M. This has the effect of preventing the degradation of penicillenic acid; thus the absorbance at 350 nm rises to a maximum and stays there. Only one rate constant (k_{Hg}) can be derived. The results are summarised in table 20.

Table 20. Reaction of phenylpenicillin (0.02g l^{-1}) at 30°C.

pH	$10^5 k_{Hg}$ /s^{-1}	A_{max}	pH	$10^5 k_{Hg}$ /s^{-1}	A_{max}
1.02	1950	1.13	2.23	170	2.20
1.14	1600	1.25	2.56	125	2.20
1.42	720	1.50	2.72	100	2.20
1.57	550	2.00	2.83	90.5	2.20

These values should represent the rates of reaction of phenylpenicillin. As expected, they are close to the values of k_1 in the low pH range, and intermediate between k_1 and k_2 in the range 2-3.

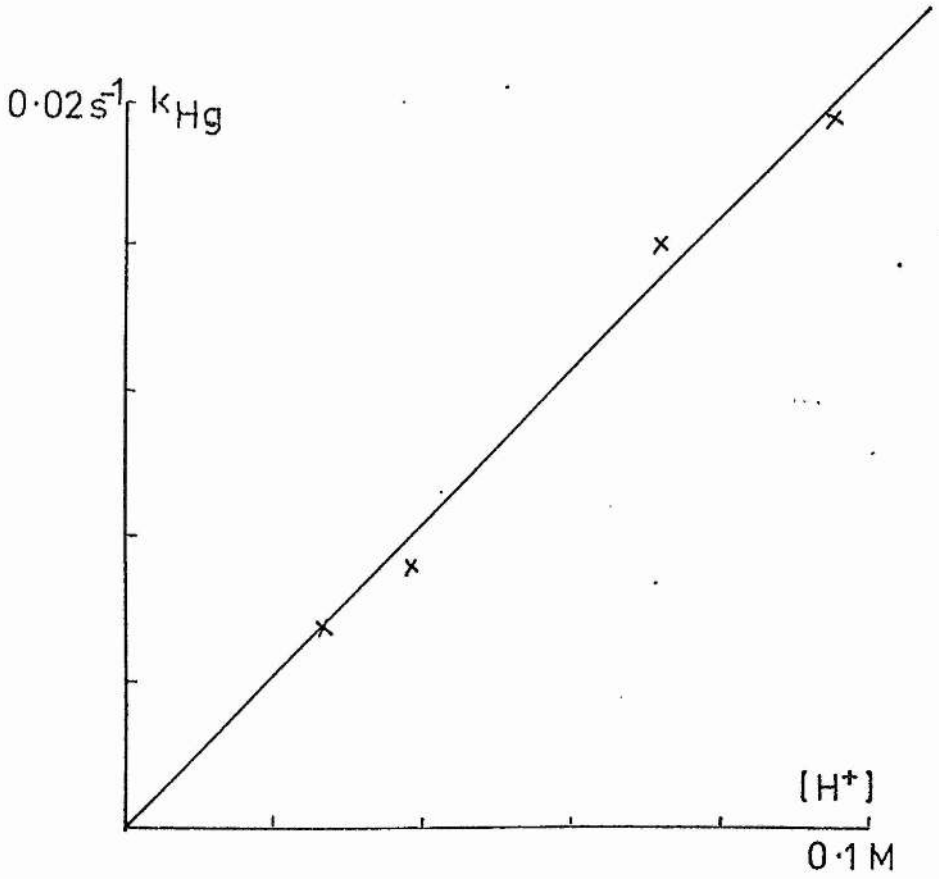


FIGURE 6: Reaction of phenylpenicillin with acid at 30°C

Figure 6 is a plot of k_{Hg} vs $[H^+]$. The gradient of the line is $0.21 \pm 0.02\ M^{-1}s^{-1}$. This may be taken as the second-order rate constant for the acid-catalysed reaction of phenylpenicillin free acid.

In order to arrive at more precise values for the rate constants, a FORTRAN computer program was devised which would calculate the values which best fitted the experimental data. Details of this program are given in Appendix 4. Briefly, the computer is given details of the optical density achieved at various times. Using this data, it works out the values of three parameters: the rate constants for the formation and decay of the intermediate (penicillenic acid), and a parameter E. This is the product of the extinction coefficient for penicillenic acid, the initial concentration of penicillin in the buffer, and the fraction of this which is converted to penicillenic acid.

For example, the data from an experiment conducted at pH 1.07, when analysed by the Swinbourne method, gave $k_1 = 2.26 \times 10^{-2}\ s^{-1}$ and $k_2 = 1.08 \times 10^{-3}\ s^{-1}$. When analysed by the computer, it gave the rate constant for the formation of the intermediate (k_a) as $1.86 \times 10^{-2}\ s^{-1}$ and the rate constant for the reaction of the intermediate (k_b) as $1.15 \times 10^{-3}\ s^{-1}$, with E = 1.68. The extinction coefficient is 24,300 and the initial concentration of penicillin was 1.1×10^{-4} M. Therefore, from the value of E, it may be estimated that the conversion to penicillenic acid was 63.5%. This example represents a relatively straightforward case, and it confirms what was known already: namely that, under these conditions, k_2 is quite a good approximation to the real value, while k_1 is about 20% too high. It was obvious that k_a had to be much greater than k_b; and with this constraint, the computer was able to find a unique set of parameters to fit the data.

The program was employed to analyse the data for experiments conducted between pH 2.27 and 3.66. The results from Swinbourne analyses have been reported earlier in table 19. Unfortunately, these experiments proved to be more complicated than the example given above. Where k_a and k_b are expected to be close in value, it was possible for the computer to find an infinite variety of sets of results, each of which fit the data fairly well. In order to find which of these possible results is the correct set, it was necessary to put an extra constraint on the program.

The constraint chosen was to make an estimate of E, make this a constant, and allow the program to find values of k_a and k_b which are consistent with this value.

In table 20 , the value of A_{max} remains constant for pH $>$ 2. This means that E must be constant within this range. (Presumably it is constant because the conversion to penicillenic acid has reached 100%) A value of 3.7 was decided on, since this gave values of k_a and k_b which were in good agreement with k_{Hg} in table 20. The values of k_a and k_b calculated by the computer are listed in table 21. The Corr. value is a measure of how closely the calculated constants fit the experimental data. It is the sum of the squares of the differences between calculated and observed optical densities over 20 data points. The lower its value, the better the results.

The values of k_a in table 21 are combined with the values of k_{Hg} in table 20 to give the pH-rate profile shown in figure 7. This has the same shape as the profile for benzylpenicillin (page 7). The kink in the curve arises because the penicillin anion reacts faster than the free acid.

The pH-rate profile for phenylpenicillenic acid is shown in

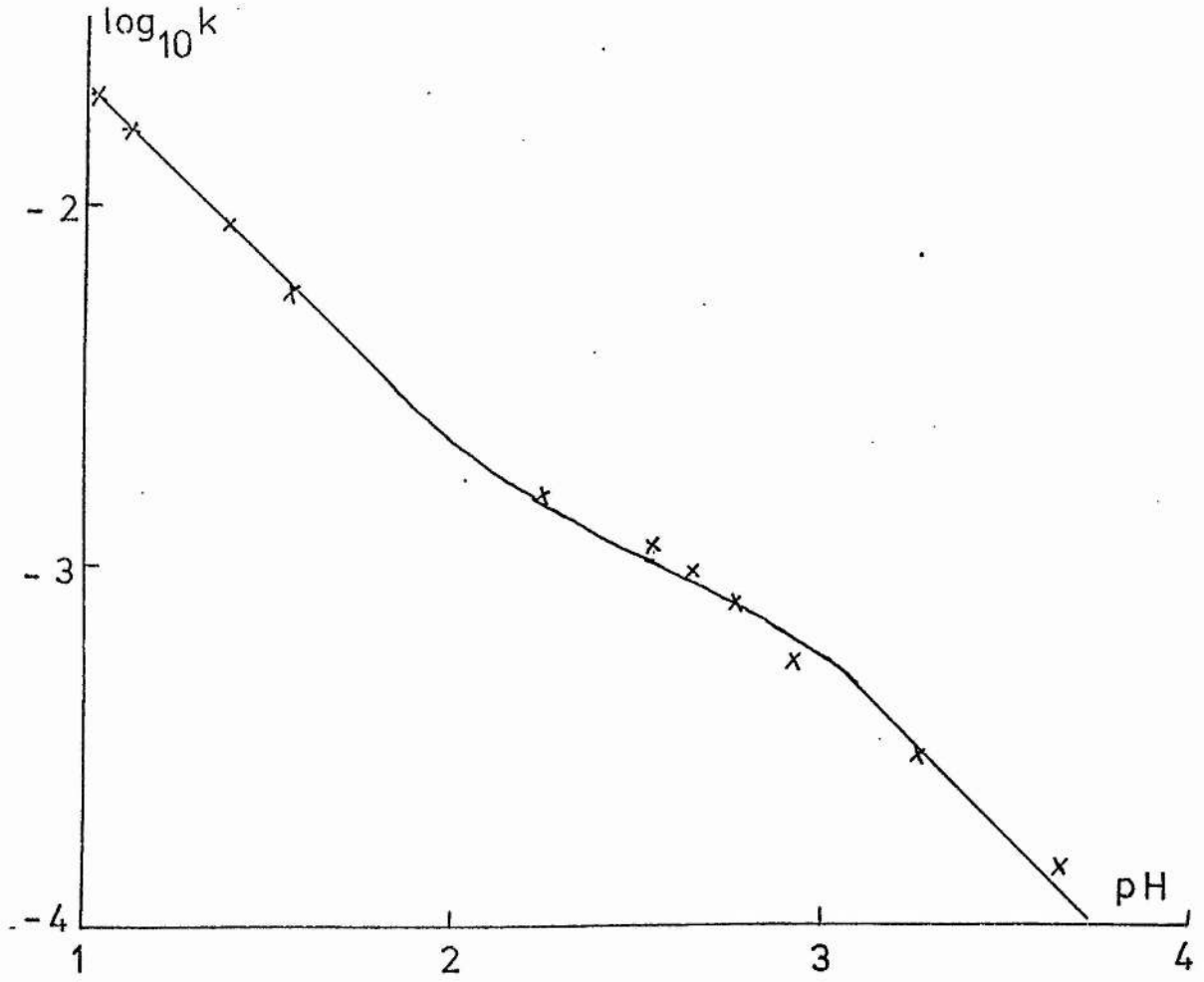


FIGURE 7. pH—rate profile for reaction of phenylpenicillin with acid at 30°C

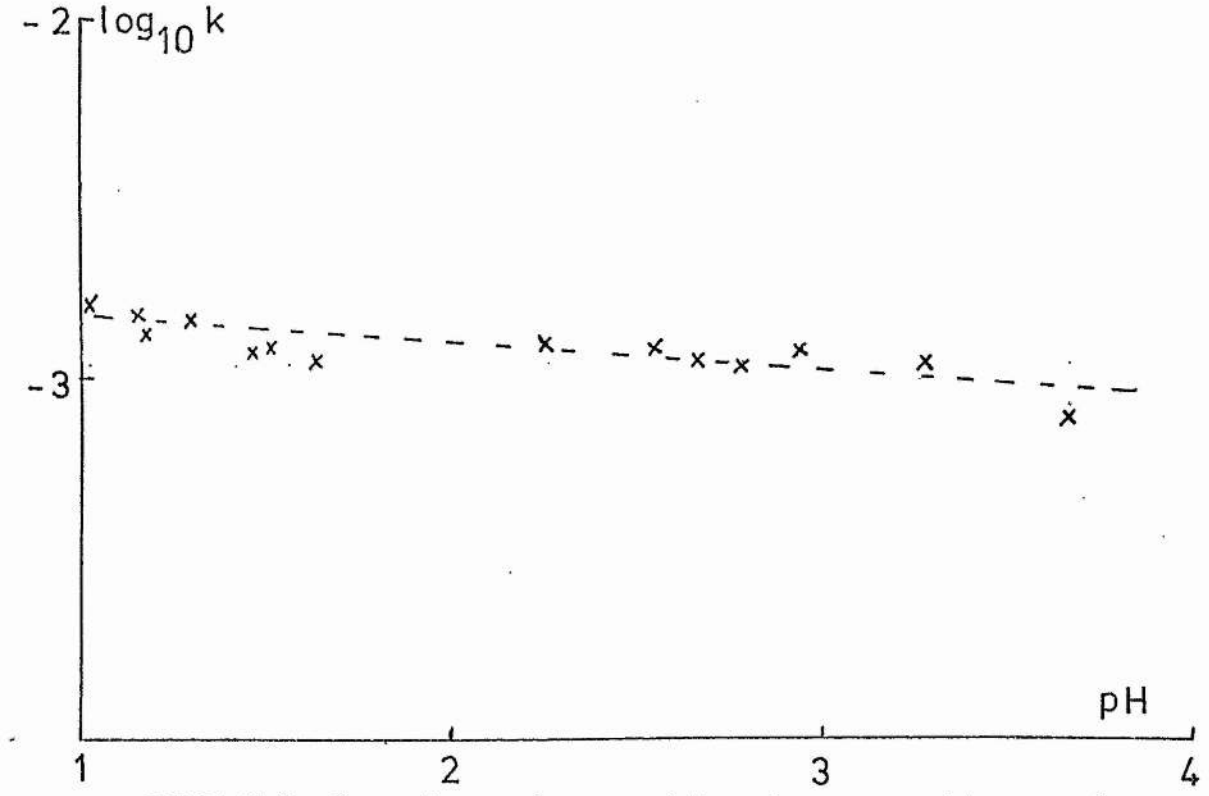


FIGURE 8 : pH—rate profile for reaction of phenylpenicillenic acid with acid at 30°C.

figure 8. It is apparent that this substance, unlike the benzylpenicillenic acid, is not very sensitive to acid. It degrades at about the same rate over a wide pH range, the rate constant being ca. 10^{-3} s^{-1}.

Table 21. Reaction of phenylpenicillin at 30°C. Computer-calculated constants.

pH	$10^5 k_a$ /s^{-1}	$10^5 k_b$ /s^{-1}	10^4 Corr.
2.27	158	125	12.1
2.56	111	116	7.64
2.67	93.9	111	8.93
2.79	75.9	104	7.76
2.94	52.5	115	30.0
3.28	29.3	107	23.4
3.66	14.1	77	1.85

p-NITROPHENYLPENICILLIN

The reaction of p-nitrophenylpenicillin in acid was studied by observation of absorbance changes at 370 nm. Rate constants were calculated for the rising (k_1) and falling (k_2) parts of the graph. The results are presented in table 22, and are illustrated in figure 9.

As in the case of phenylpenicillin, the maximum absorbance decreases dramatically as the pH is raised; and again this is attributed to a reversal of the relative rates of the two processes being observed. In this case the results are only meaningful for pH > 2. Below this, k_1 and k_2 are too close together.

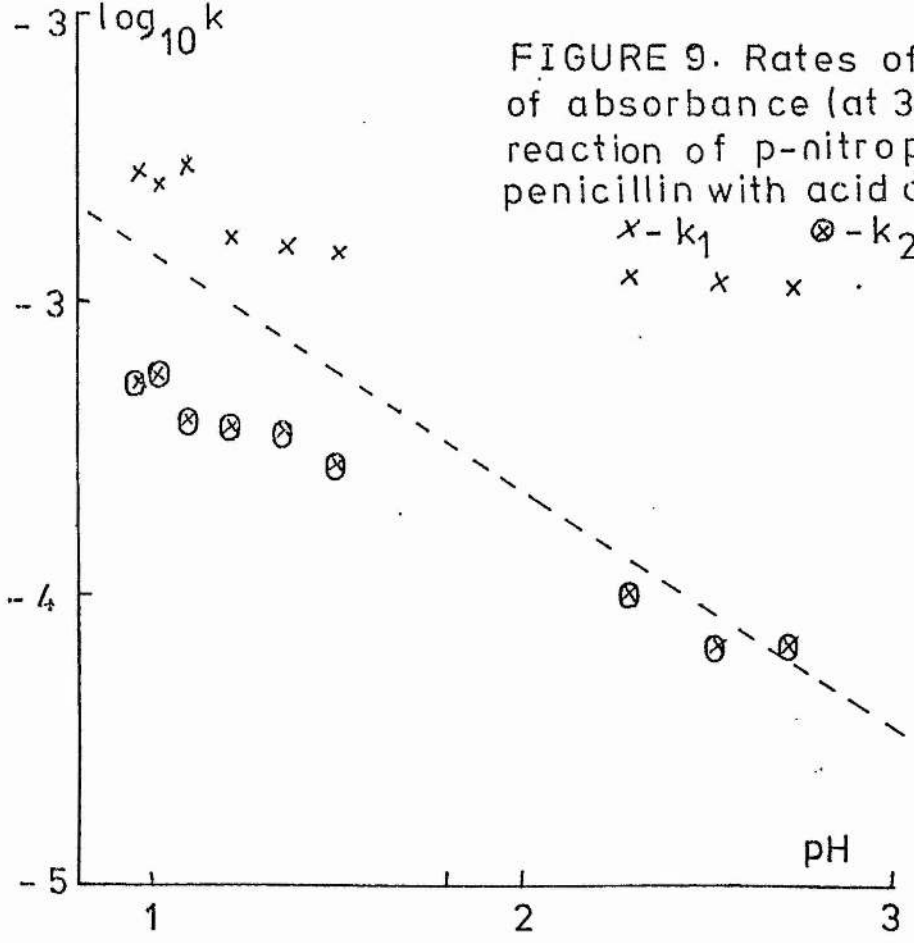


FIGURE 9. Rates of change of absorbance (at 370 nm) in reaction of p-nitrophenyl-penicillin with acid at 30°C.
× - k_1 ⊗ - k_2

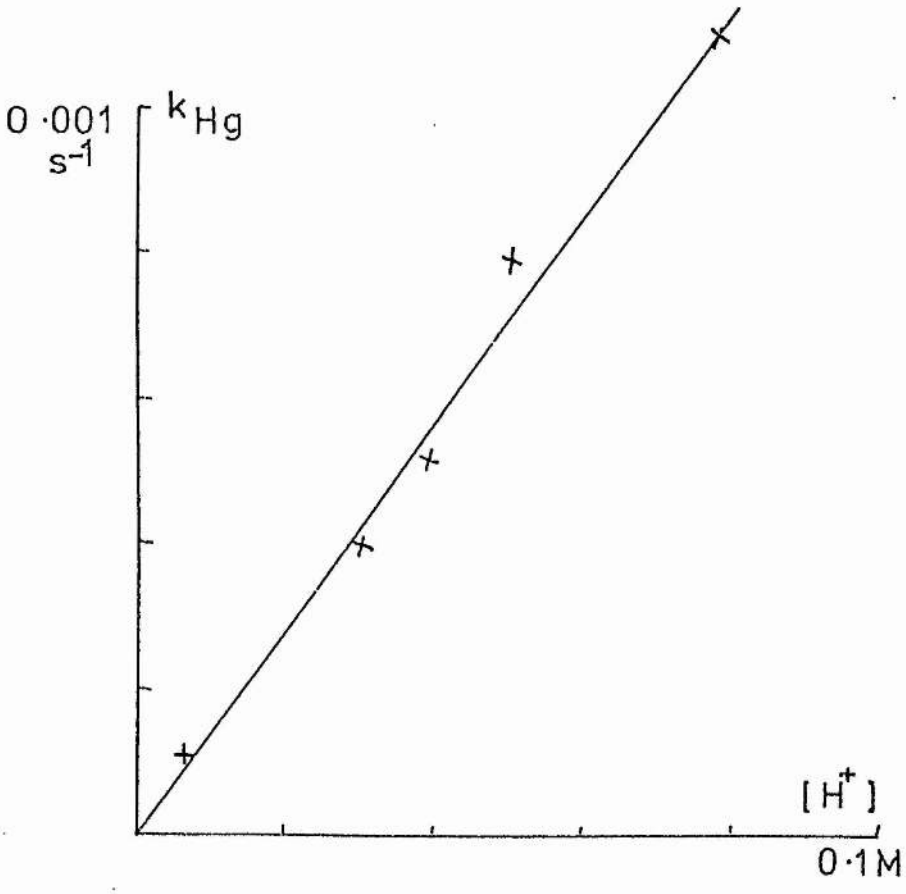


FIGURE 10. Reaction of p-nitrophenylpenicillin with acid at 30°C.

Table 22. Reaction of p-nitrophenylpenicillin (0.04 g l^{-1}) with acid at 30°C.

pH	$10^5 k_1$ /s^{-1}	$10^5 k_2$ /s^{-1}	A_{max}
0.97	280	52.5	1.18
1.01	260	55.5	1.22
1.10	295	40.0	1.16
1.22	170	38.5	0.99
1.35	155	36.5	0.90
1.49	150	29.0	0.76
2.29	125	10.5	0.35
2.52	120	6.8	0.29
2.70	115	7.0	0.26

Once again, the experiments were performed in buffers containing $HgCl_2$; and the results obtained are given in table 23.

Table 23. Reaction of p-nitrophenylpenicillin with acid at 30°C.

pH	$10^5 k_{Hg}$ /s^{-1}	A_{max}	pH	$10^5 k_{Hg}$ /s^{-1}	A_{max}
1.10	110	1.20	2.54	6.7	1.28
1.30	79.5	1.44	2.73	6.7	1.14
1.41	52.0	1.20	2.84	4.85	0.65
1.52	40.0	1.30	3.18	4.20	0.46
2.22	10.5	1.56			

As expected, the values of k_{Hg} are intermediate between k_1 and k_2 at pH < 2; and they are practically identical with k_2 for pH > 2. Therefore k_{Hg} may be taken as the true value for the reaction of p-nitrophenylpenicillin. It is plotted as a function of $[H^+]$ in

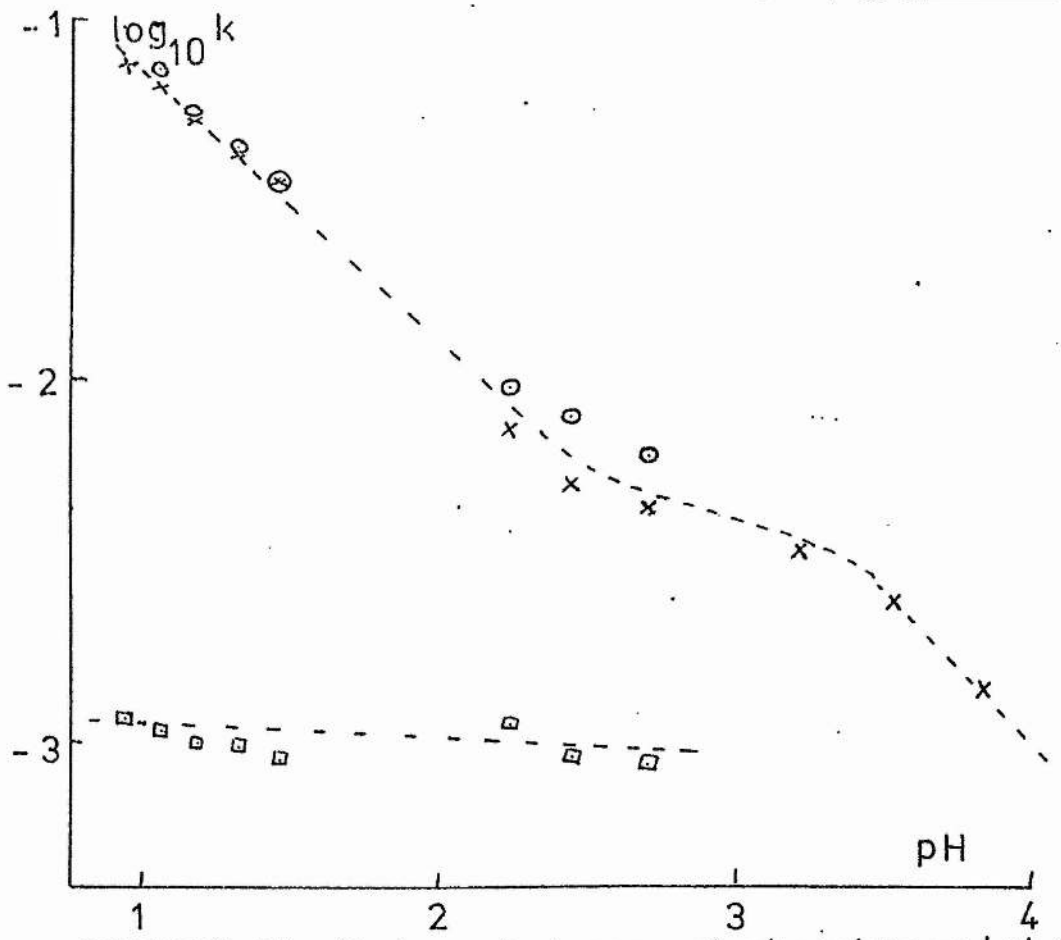


FIGURE 11. Rates of change of absorbance (at 360 nm) in reaction of p-methoxyphenylpenicillin with acid at 30°C
o - k_1 □ - k_2 x - k_{Hg}

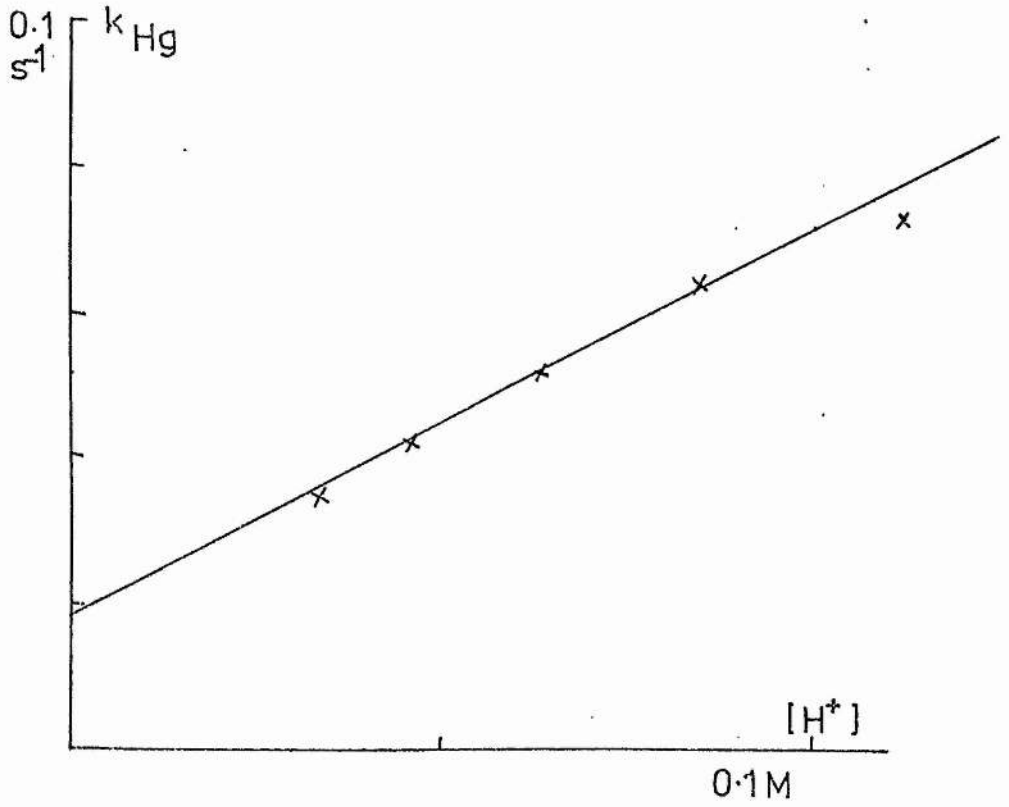


FIGURE 12. Reaction of p-methoxyphenylpenicillin with acid at 30°C.

figure 10. The gradient of the straight line is $0.014 \pm 0.002\ M^{-1}s^{-1}$. This, therefore, is the second-order rate-constant for the reaction of p-nitrophenylpenicillin free acid. At pH > 2, the points lie considerably above this line. This is because, in this pH range, significant amounts of p-nitrophenylpenicillin anion are present. This reacts faster than the free acid.

The rate-constants for the reaction of p-nitrophenylpenicillenic acid cannot be known with certainty, but a perusal of the above results would suggest that these are not greatly affected by the acidity of the solution, and have values of $1\text{-}2 \times 10^{-3}\ s^{-1}$ throughout the range studied.

p-METHOXYPHENYLPENICILLIN

The reaction of p-methoxyphenylpenicillin was studied by observation of absorbance changes at 360 nm. Buffers both with and without $HgCl_2$ were employed, and values for k_1, k_2 and k_{Hg} were obtained as before. They are summarised in table 24.

Table 24. Reaction of p-methoxyphenylpenicillin at 30°C.

pH	$10^5 k_1\ /s^{-1}$	$10^5 k_2\ /s^{-1}$	$10^5 k_{Hg}\ /s^{-1}$
0.95	9300	115	7300
1.07	7100	105	6400
1.19	5400	99	5200
1.33	4450	96.5	4150
1.47	3600	87.5	3500
2.25	945	110	710
2.46	780	91	510
2.71	605	84	415

Table 24 - continued.

pH	3.23	3:54	3.84
$10^5 k_{Hg}/s^{-1}$	335	245	140

These results are illustrated in figure 11. The value of k_{Hg} can be taken as representing the rate of transformation of the penicillin into penicillenic acid, and it is in reasonable agreement with k_1 throughout the range studied. The value of k_2, therefore, represents the rate at which the penicillenic acid reacts. This is considerably lower than the rate at which it is formed throughout this range. As in the case of phenyl- and p-nitrophenyl- penicillin, this rate appears to be almost invariant with pH, and k is ca. $10^{-3} s^{-1}$.

When k_{Hg} is plotted against $[H^+]$ (figure 12), a straight line is obtained for pH < 2. This line, however, does not pass through the origin, but has an intercept of $0.0185\ s^{-1}$. Its gradient is $0.53 \pm 0.045\ M^{-1}s^{-1}$. Points for pH > 2 lie considerably below this line. The significance of these findings will be discussed later.

METHICILLIN

The reaction of methicillin was studied by observation of the absorbance changes at 330 nm. The results from buffers both with and without $HgCl_2$ are summarised in table 25, and are illustrated in figure 13.

This case is similar to that of p-nitrophenylpenicillin. The value of k_{Hg} may be taken as the rate of reaction of methicillin. These values are intermediate between k_1 and k_2 at low pH; but above pH 2 they are very close to k_2. For pH < 2, a plot of k_{Hg} against $[H^+]$ gives a straight line (figure 14) which passes through the

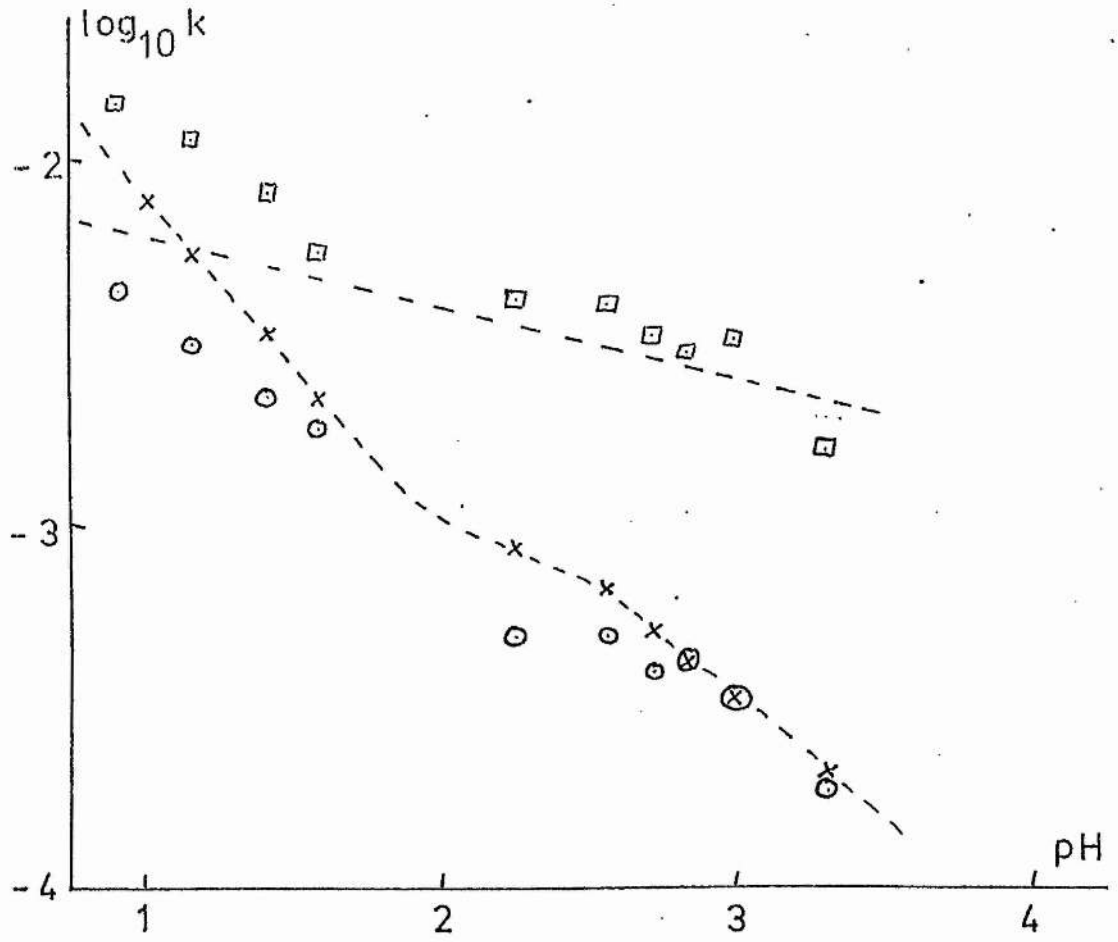


FIGURE 13. Rates of change of absorbance (at 330 nm) in reaction of methicillin with acid at 30°C.

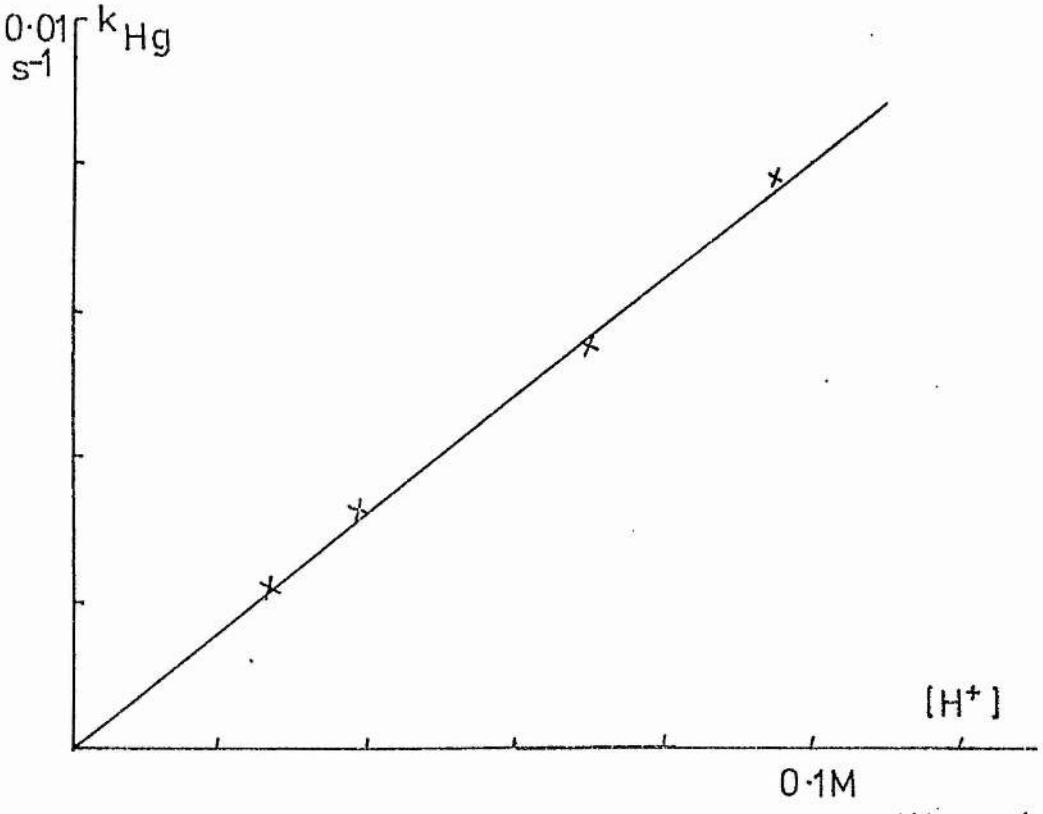


FIGURE 14. Reaction of methicillin with acid at 30°C.

Table 25. Reaction of methicillin in acid at 30°C.

pH	$10^5 k_1 / s^{-1}$	$10^5 k_2 / s^{-1}$	$10^5 k_{Hg} / s^{-1}$
0.91	1450	445	
1.02			780
1.15	1150	310	550
1.41	815	220	330
1.57	565	185	220
2.24	420	87	87
2.56	405	50	65
2.72	330	39	51
2.83	295	41	43
2.99	318	32.5	33
3.30	160	18.5	21
3.66			14

origin. The gradient is 0.0805 ± 0.0045 $M^{-1}s^{-1}$, and this may be taken as the second-order rate constant for the reaction of methicillin free acid.

At pH > 2, k_1 ought to be a good approximation for the rate of reaction of the penicillenic acid. At lower pH, it may be guessed that this reacts at about the same rate as it is formed. Thus in the range studied it appears to be rather more reactive than the other phenylpenicillenic acids encountered, though not so reactive as benzylpenicillenic acid. Further, its reaction rate is more sensitive to changes in the pH, though it is far from being directly proportional to $[H^+]$, as is the rate for benzylpenicillenic acid.

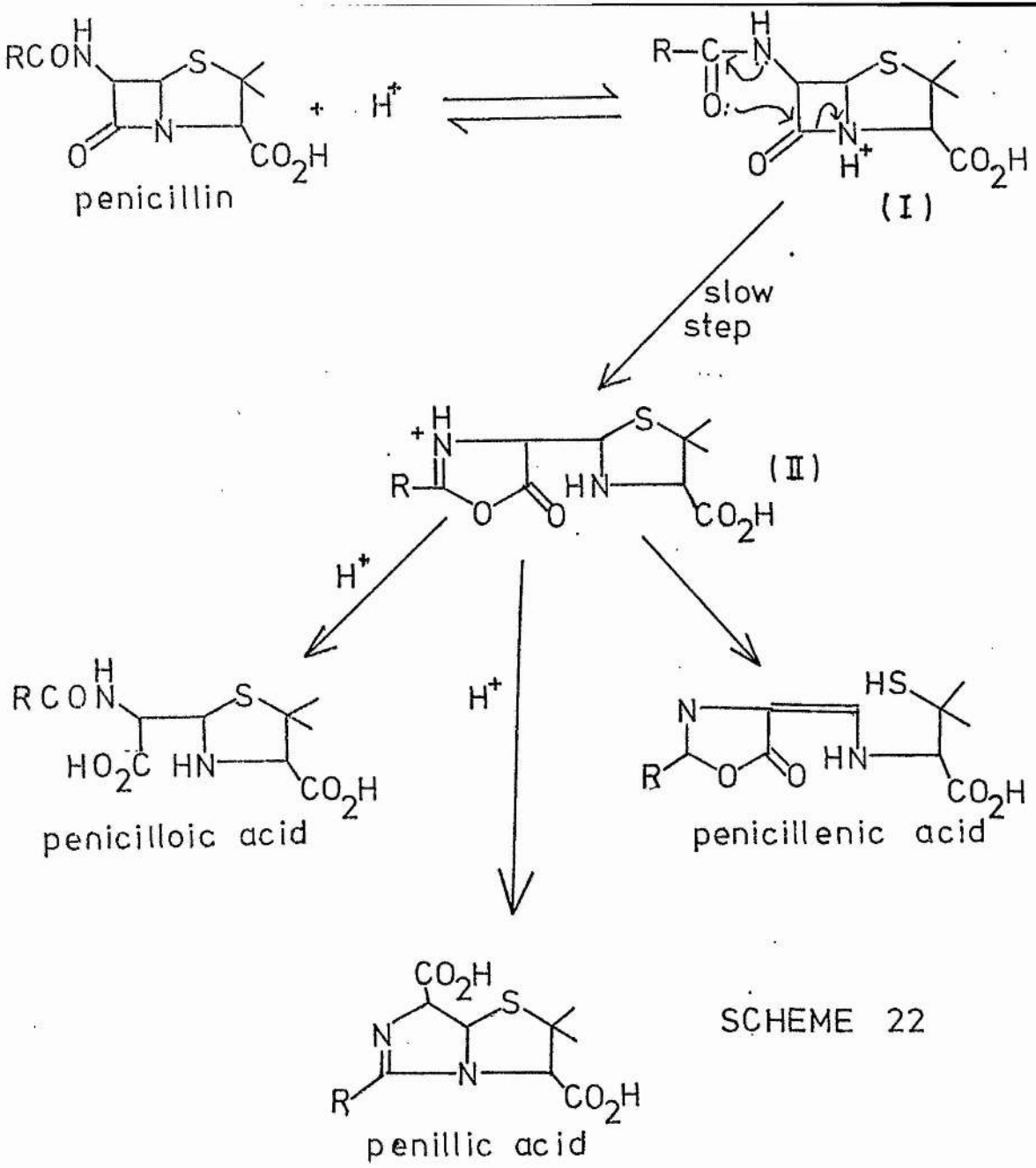
DISCUSSION

Table 26 summarises the second-order rate constants for the reactions of all the penicillins so far studied with acid.

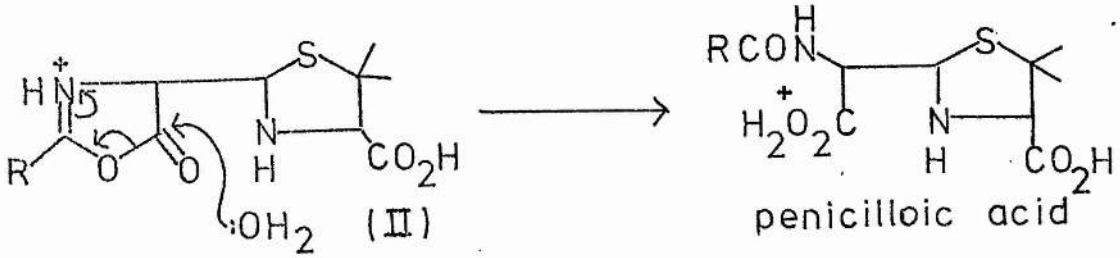
Table 26. Reaction of penicillins with acid at 30°C.

Penicillin	k_2 /$M^{-1}s^{-1}$
benzyl-, free acid	0.095
, anion	0.375
, free acid, in D_2O	0.235
phenyl, free acid	0.21
p-nitrophenyl-, free acid	0.014
p-methoxyphenyl-, free acid	0.53
methicillin, free acid	0.0805

These results are consistent with the reaction mechanism illustrated in scheme 22, which incorporates the processes originally suggested by Woodward et al.[47] in 1949. It is suggested here that the same scheme operates for both the free acid and the anion form of the penicillin. This is contrary to the view of Schwartz[42], who suggested that these species give rise to different products in solution: the anion to penicillenic acid, and the free acid to penicilloic acid. This theory was invoked to explain why the proportions of these two products changed so substantially with the pH. It is, however, difficult to envisage why there should be such a difference between the reactions of the anion and its conjugate



SCHEME 22



SCHEME 23

acid. The mechanism proposed here satisfactorily explains the changing proportions of the products, while avoiding the above-mentioned difficulty.

Protonation of the penicillin by the acid leads to the formation of a small amount of (I), which is the reactive species. It then undergoes a rearrangement, initiated by nucleophilic attack on the β-lactam by the side-chain, and leading to intermediate (II). This step is normally the rate-limiting process for the reaction. (II) reacts faster than it is formed, and is transformed into either penicilloic, penicillenic or penillic acid. The latter two transformations are illustrated by schemes 5 and 6 on page 9. Hydrolysis to penicilloic acid is illustrated by scheme 23. It is suggested that the penicillenic acid transformation is a completely spontaneous process, the rate of which is independent of the hydrogen ion concentration; however the other two are catalysed by acid. These two become progressively slower as the pH is increased, but penicillenic acid continues to be formed at the same rate. This is reflected in the changing preponderances of the three products illustrated by table 13, page 67.

The product-determining steps occur relatively quickly. However, the overall rate of reaction is determined by the amount of protonated penicillin (I) available, and by how quickly this can rearrange to (II). D_2O is a weaker base than H_2O; therefore when the reactions are performed in D_2O buffers, the concentration of (I) is substantially increased. This is reflected in an increased rate of reaction. The equilibrium concentration of (I) is also increased if the penicillin exists in its anion form rather than the free acid form. Thus the second-order rate constant is higher in the higher pH range.

Confirmation that the rearrangement of (I) is rate-determining is afforded by observing the manner in which the side-chain affects the reaction rate. When the benzyl group is replaced by phenyl the rate doubles. This is because the side-chain carbonyl, in direct conjugation with the benzene ring, withdraws negative charge from that ring and thus becomes more nucleophilic. This effect is reversed when a second electron-withdrawing group ($-NO_2$) is attached to the opposite end of the benzene. The p-NO_2 group pulls charge away from the carbonyl, thus making it less nucleophilic. CH_3O- is a strongly electron-donating substituent, so the p-methoxyphenyl group makes the carbonyl even more nucleophilic than before. It might have been expected that methicillin, with its two methoxy groups, would be the most reactive of all the phenylpenicillins; but its rate constant is very similar to that of benzylpenicillin. This may be attributed to steric hindrance. Further examples of side-chain effects on the reaction rate are the cases of ampicillin and penicillin V. These are particularly noted for their stability towards acids; and their resistance may be attributed to the presence of an electronegative atom close to the side-chain carbonyl, whose nucleophilicity is therefore much reduced. (At this point, it may be intructive to compare the action on various penicillins of CH_3I /Ag_2O. This was discussed in chapter 2. The ease with which the β-lactam nitrogen is methylated also seems to be affected by the nucleophilicity of the side-chain.)

In this proposed mechanism, penicilloic acid is thought to arise from hydrolysis of (II), rather than by direct acid-catalysed hydrolysis of the penicillin itself. Direct hydrolysis of the penicillin ought to occur by the same mechanism as the acid-catalysed

hydrolysis of 6-APA. It is known[43] that 6-APA, in common with most amides and, indeed, most β-lactams[84], hydrolyses extremely slowly in acid. If the production of penicilloic acid were solely dependent upon the same mechanism, it would take place at a rate far slower than the production of other products; and so it could not be formed in the proportions which it evidently is formed in (45%). Any explanation of why the penicilloic acid of a penicillin forms substantially more quickly than that of 6-APA must postulate some involvement of the side-chain; and the route via intermediate (II) is the most obvious suggestion.

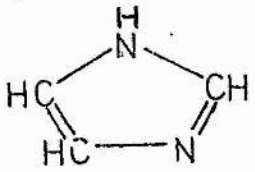
Before leaving this matter, the case of p-methoxyphenylpenicillin requires some further comment. Results at the low pH range strongly suggest that there is a non-catalysed reaction taking place at a significant rate, as well as a catalysed one. Also, as the pH is raised above 2, the reaction is slower than would be predicted by extrapolation of the results from $pH < 2$. In both these respects the behaviour is markedly different from that of any other penicillin studied in this work. The rate-enhancement caused by the p-methoxy substituent is 2.5; but a consideration of linear free energy relationships[83] would suggest that it ought to be larger than this. σ_{p+} for $-NO_2$ and $-OCH_3$ are 0.78 and -0.79 respectively. A possible explanation for the strange behaviour of this penicillin is that the p-OCH_3 substituent causes (I) to rearrange so fast that this rearrangement ceases to be the rate-determining step. The reaction of (II) then becomes rate-determining. (The rate at which (II) reacts is unlikely to be much affected by the nature of the side-chain.) As suggested above, one of the three possible reaction pathways for (II), its conversion to penicillenic acid, is not acid-catalysed.

The value of 0.0185 s^{-1} would represent the rate of this reaction throughout the pH range. It is the minimum rate at which (II) reacts. In the higher pH range, though, the overall reaction does proceed slower than this. This would be because, with a lower hydrogen ion concentration, the production of (II) becomes slower than its reaction; ie there is a change in the rate-determining step.

Finally, some remarks concerning the stability of the penicillenic acids. It is evident that the phenyl compounds enjoy a far greater stability than the benzyl compound. This can be attributed to the extra conjugation between the phenyl and oxazolone rings. Because of this extra conjugation, the transformation of (II) into penicillenic acid occurs at a faster rate; and therefore it is formed in greater abundance than is benzylpenicillenic acid throughout the acid pH range. Once formed, it is also slower to react. It is not that phenylpenicillenic acids are less reactive than benzylpenicillenic acid generally (because both disappear fairly quickly in neutral aqueous solution); it is rather that the phenyl compounds are curiously insensitive towards acid. A brief perusal of the way the products change with pH[35] would suggest that the mechanism of degradation of benzylpenicillenic acid was a pH-independent rearrangement to penillic acid combined with an acid-catalysed hydrolysis to penamaldic acid. In the case of phenylpenicillenic acid, it appears that this latter reaction is missing, since penamaldic acid cannot be detected among the products, and the entire reaction is virtually independent of pH. Why this should be so is difficult to say.

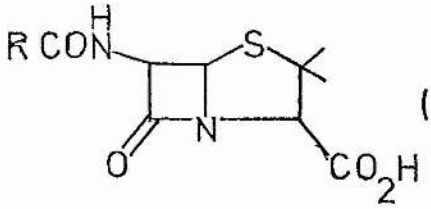
CHAPTER FOUR

A KINETIC STUDY OF THE REACTION OF BENZYLPENICILLIN WITH IMIDAZOLES



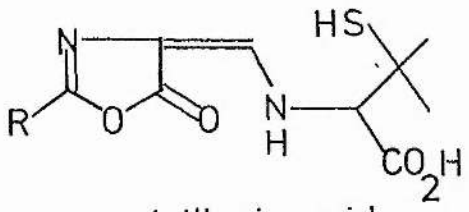
(43)

imidazole



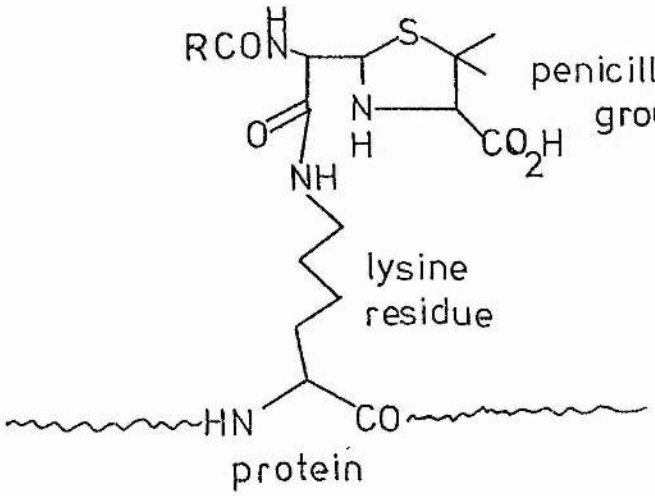
(3)

penicillin



(28)

penicillenic acid



penicilloyl group

lysine residue

protein

(44)

INTRODUCTION

In recent years there has been interest in the reaction between penicillins and the heterocyclic base imidazole (43). This was first investigated by Grant et al.[85], and more recently the matter has been taken up by Bundgaard[86]. There are two reasons why this reaction should be of particular interest.

Firstly, it provides a convenient and quick method of penicillin assay by spectrophotometric analysis[75]. This is because imidazole catalyses the transformation of penicillins (3) into the corresponding penicillenic acids (28) in virtually quantitative yield. The intense ultraviolet absorbance associated with penicillenic acids allows their concentrations to be determined accurately and related to the original concentration of penicillin. In these experiments, the extremely labile penicillenic acids are stabilized in solution by the presence of an equivalent amount of mercury(II) ion. Although penicillenic acid is also formed by the reaction of penicillin with aqueous acid, it is in this case only one of several products; so this reaction cannot form the basis of an assay. In the imidazole-catalysed reaction, it appears that penicillenic acid is the sole product.

Second, there has been a growing interest in the reactions of penicillins with amines generally. This is related to an interest in the nature of penicillin allergies. The principle antigenic determinant of penicillin allergy is thought to be a penicilloyl group bound by an amide linkage to the ε-amino group of a lysine residue in protein (44)[87, 88, 89]. This could arise either from

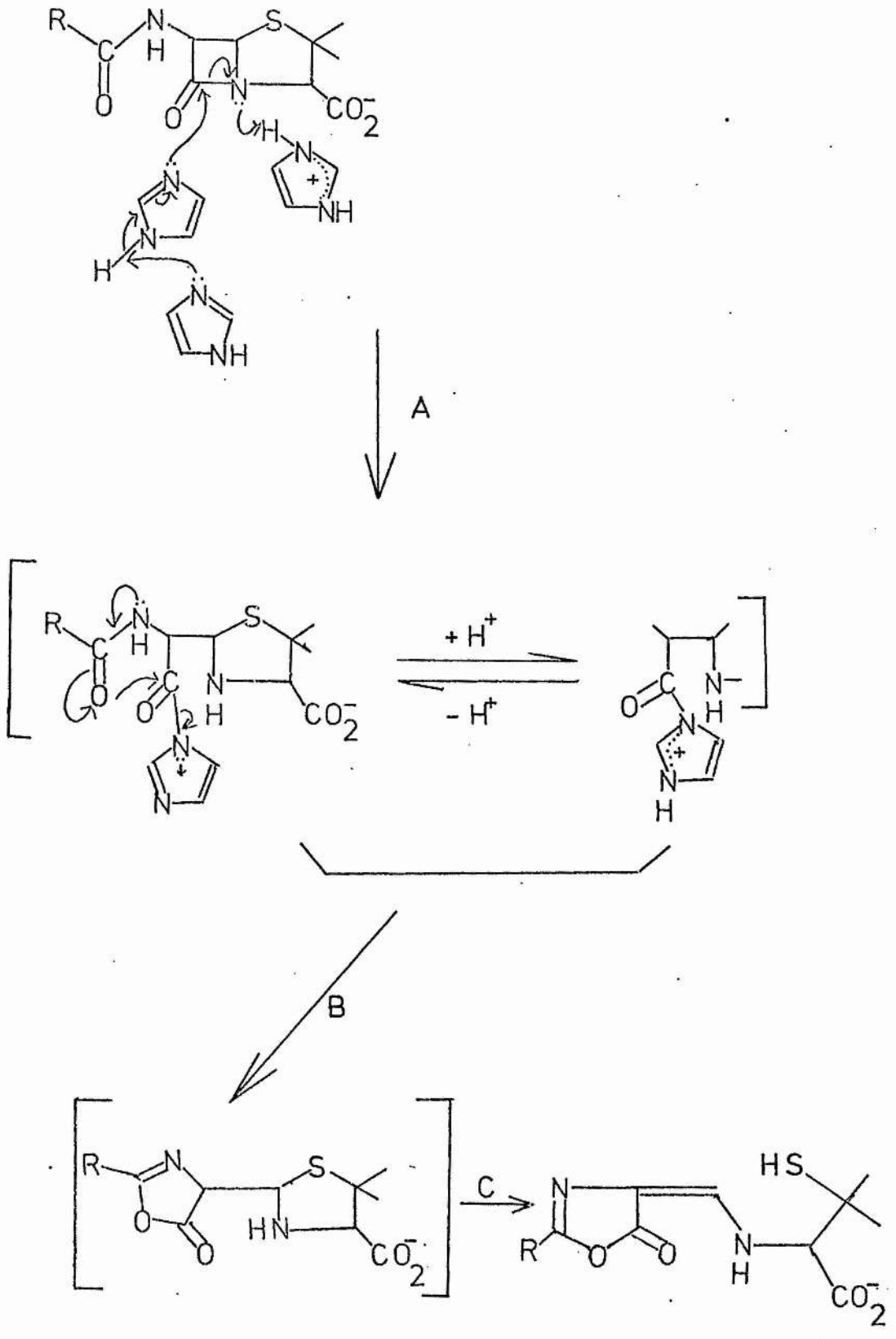
direct aminolysis of the penicillin *in vivo*, or by aminolysis of the more reactive penicillenic acid. *In vitro* studies have shown that both aminolysis of penicillin and its transformation to penicillenic acid are exceedingly slow processes at a physiological pH. However, there is reason to suppose that these reactions can proceed much faster in living systems. It has, for example, been shown[45] that copper(II) ions (which are present in human serum to the extent of 0.016 mM) increase the rate of aminolysis of penicillin G by a factor of up to 10^7. Also, even at neutral pH, the imidazole-catalysed transformation to penicillenic acid is relatively fast. (It proceeds faster than most aminolyses at neutral pH partly because of imidazole's low basicity. Its pK_a at 35°C is 6.96, compared with 9.41 for glycine and 10.55 for lysine.)

Imidazole is present in living systems, incorporated in the histidine residues of protein. This biological imidazole is thought to play an important role in the action of enzymes such as chymotrypsin, which cleave the amide linkages of proteins[90]. In its reaction with penicillin, it is again an amide linkage which is cleaved.

Bundgaard[86] has studied the kinetics of the reaction of benzylpenicillin with imidazole. He finds that, with imidazole in excess, the reaction is first-order with respect to penicillin. However, the first-order rate constants are not directly proportional to imidazole concentration, but rather follow the relationship (i).

$$k_{obs} = k_1 [\,Im\,][\,ImH^+\,] + k_2 [\,Im\,]^2 \qquad -(i)$$

Here $[\,Im\,]$ is the concentration of imidazole free base and $[\,ImH^+\,]$ the concentration of its conjugate acid, the imidazolium ion.



SCHEME 24

(Other workers[91] do claim to have observed a third term in this rate expression, of the form k_3 [Im] . However, this term is insignificant in comparison with the first two.

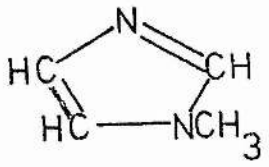
These terms in the rate equation are interpreted by Bundgaard as arising from nucleophilic attack of imidazole free base on the β-lactam carbonyl of the penicillin. This is assisted both by general base catalysis from another imidazole molecule (k_2 term) and by general acid catalysis from an imidazolium ion (k_1 term). This is summarised in scheme 24 step A. The resulting penicilloyl-imidazole then decomposes as a result of an intramolecular nucleophilic attack, leading to expulsion of imidazole and formation of an oxazolone-thiazolidine structure (step B). This, as has been observed previously, is unstable and rearranges to penicillenic acid (step C).

In the case of most aminolysis reactions of penicillin, the ultimate product is the penicilloylamide[92]. In other words, the reaction would cease after the equivalent of step A. However, N-acylimidazoles are known to be far more susceptible to nucleophilic attack than conventional amides[93]. (This is because the electron pair from nitrogen, which is normally involved in resonance with the carbonyl group, is part of the aromatic imidazole ring. The pK_a of acetylimidazole, for example, is 3.8, compared with values less than zero for ordinary amides.) Thus step B takes place easily; it is entirely analogous to the intramolecular attack postulated earlier for the acid-catalysed reaction of penicillins.

Step A is envisaged as being rate-determining. But although this step incorporates all the ideas necessary to explain the observed rate law, it does not satisfactorily account for it on its own.

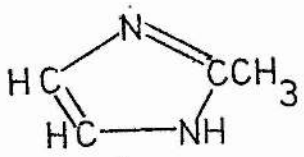
Firstly, the tetramolecular collision envisaged here would be impossible. Second, the type of general base catalysis depicted does not make sense in this context. Such a scheme (involving two imidazole molecules) has been postulated in the past[94] to explain a term in $[\text{Im}]^2$ in the imidazole-catalysed hydrolysis of esters with poor leaving groups (such as p-methylphenyl acetate); however in that case there was no dependence of rate on $[\text{ImH}^+]$. The fact that $[\text{ImH}^+]$ does appear in the rate expression for the reaction with penicillin means, for reasons which will be explained later, that proton transfer must be the slow step, and nucleophilic attack by imidazole on penicillin must be faster. Increasing the nucleophilicity of imidazole, by interaction with a second imidazole molecule, must make this step faster still; but since the term in question depends only on $[\text{Im}]^2$, this step would have to be rate-determining. It cannot speed up and become the slow step at one and the same time.

A more detailed description of this part of the mechanism is therefore called for. To this end, it was decided to embark on a wider investigation of the effect of imidazole on benzylpenicillin. After repeating the original work of Bundgaard, a determination of solvent kinetic isotope effects was undertaken. After this, substituted imidazoles were investigated. Of particular interest was the effect of using N-methylimidazole (45), as this would be unable to provide general base catalysis of the type envisaged is step A. It was hoped that these studies would shed more light on the mechanism.



(45)

N--methylimidazole



(46)

2-methylimidazole

EXPERIMENTAL

A. MATERIALS

The imidazole was specially purified by its manufacturer (BDH) for use in ultraviolet studies. N-methylimidazole and 2-methyl imidazole (46) were purchased from Aldrich Company, and used without further purification.

Stock solutions of the imidazole (ca. 1M) were prepared by dissolving a known weight of the substance in water, and making up to a known volume in a standard flask. The concentration was then checked by titration against a standard (1M) of hydrochloric acid, with methyl red as indicator.

Buffer solutions were prepared by suitable mixtures of the stock imidazole solutions and HCl solution. The ionic strength was adjusted to 0.5M by addition of calculated amounts of potassium chloride. All the solutions were made up with water containing 10^{-4} M of mercuric chloride.

Deuteriated buffer solutions were prepared in an analogous manner. The imidazole was first dissolved in a little D_2O, and the solution allowed to evaporate to dryness. This procedure was then repeated, to ensure complete exchange of deuterium for the labile hydrogen. The deuteriated imidazole was then made up into solution as before, and its concentration determined by titration. A 20% solution of DCl in D_2O was purchased from Aldrich Company. This was diluted to approximately 1M acid with D_2O, and the final concentration determined by titration against standard 1M sodium hydroxide solution, with phenolphthalein as indicator.

B. PROCEDURE

Rate constants were determined by following the increase in optical density at 325 nm. The procedure for this has been outlined before (page 53). In each experiment, the initial concentration of benzylpenicillin was $4 - 6 \times 10^{-5}$M. All experiments were performed at 37°C.

The pH of the buffer solutions was not measured directly. Where quoted, it has been estimated from the expression

$$pH = pK_a - \log_{10}\left([ImH^+] / [Im] \right) \quad - (ii)$$

where, at 37°C, pK_a for imidazole is 6.96, for N-methylimidazole it is 7.11 and for 2-methylimidazole it is 7.89.

RESULTS

A. PRELIMINARIES

Bundgaard[86] asserted that, in imidazole buffer solutions, benzylpenicillin is completely converted into penicillenic acid. This hypothesis was tested with the substituted imidazoles, by noting the maximum optical densities (at 322 nm) recorded for solutions of benzylpenicillin in these buffers. The results are in table 27.

Table 27. % conversion of benzylpenicillin to penicillenic acid.

Concentration of benzylpenicillin in buffer = 4.97×10^{-5}M

ε_{322} of benzylpenicillenic acid = 2.63×10^4

Buffer based on	Max. O.D. (322 nm)	% conversion of penicillenic acid
Imidazole	1.314	100.6
N-methylimidazole	1.262	96.6
2-methylimidazole	0.694	53.1

Bundgaard also asserts that the mercuric chloride present in the solutions has no effect on the rate of reaction of benzylpenicillin. The effect of varying the concentration of mercuric chloride is shown in table 28.

Table 28. Effect of $[Hg^{2+}]$ on reaction of benzylpenicillin with imidazole at 37°C.

$[Im]_t$ = 0.3M; [Benzylpenicillin] = 5.3×10^{-5}M; pH = 7.00

$[HgCl_2]$/M	Max. O.D. (325 nm)	$10^4 k_{obs}/s^{-1}$
1×10^{-3}	1.26	1.8
8×10^{-4}	1.33	1.7
6×10^{-4}	1.30	1.7
4×10^{-4}	1.29	1.7
1×10^{-5}	1.25	2.4
8×10^{-6}	1.08	2.4
4×10^{-6}	0.74	2.8

These results indicate that the concentration of Hg^{2+} does not have any effect upon the reaction rate, provided that enough is present to complex with all of the penicillenic acid. If this condition is not met, then the theoretical maximum optical density is not achieved, and the observed rate constants are higher than they should be. This apparent increase of rate constant is an artefact, arising from the degradation of uncomplexed penicillenic acid, a process which gives rise to further changes in the optical density.

It may be asked whether the spectrum of benzylpenicillenic acid mercury mercaptide is in fact the same as that of free penicillenic acid.

Table 29 details measurements which were made on solutions of authentic benzylpenicillenic acid in different solvents.

Table 29. Extinction coefficient of benzylpenicillenic acid

Penicillenic acid = 4.8×10^{-5}M

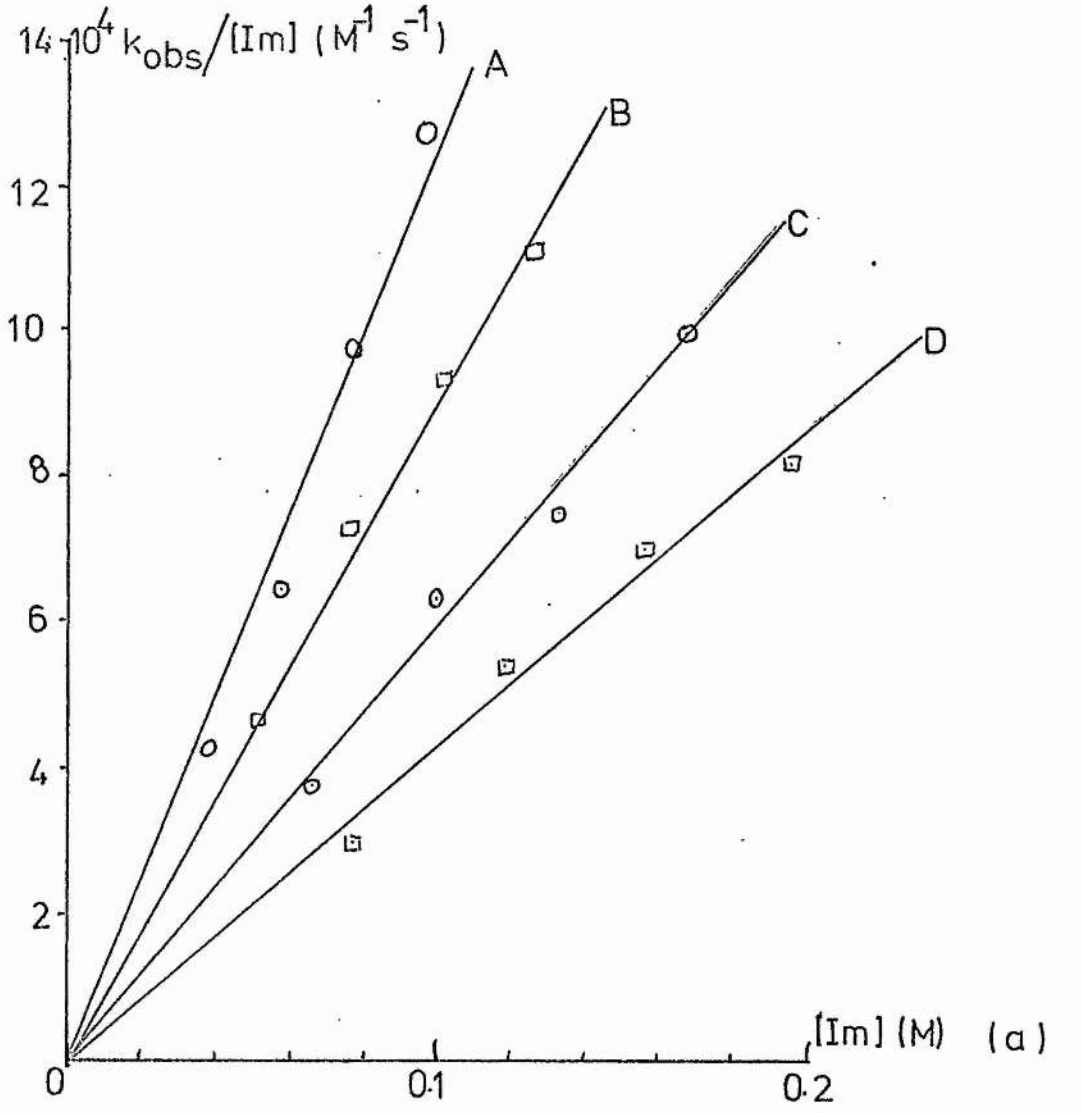
Solvent	λ_{max}	ε at λ_{max}
ethanol	322 nm	22, 300
water	320 nm	23, 400
$HgCl_2$ soln (10^{-4}M)	338 nm	16, 500
$HgCl_2$ soln (10^{-4}M) with KCl (0.5M)	322 nm	21, 300

Therefore, although the presence of Hg^{2+} ion does alter the spectrum of benzylpenicillenic acid quite considerably, this effect is not observed when potassium chloride is present.

It is known[95] that the mercury(II) ion forms a complex with imidazole.

$$Hg^{2+} + 2\ C_3H_4N_2 \rightleftharpoons [Hg(C_3H_4N_2)_2]^{2+}$$
$$K = 10^{16.7}\ M^{-2}$$

During the experiments, however, no adverse effects were detected which might arise from this complexation. However, with N-methylimidazole and 2-methylimidazole buffers, considerable precipitation occurred if the concentration of Hg^{2+} ion was raised above 4×10^{-4}M. This precipitate may be the complex with mercury of the substituted imidazole. The effect was not observed with imidazole itself.



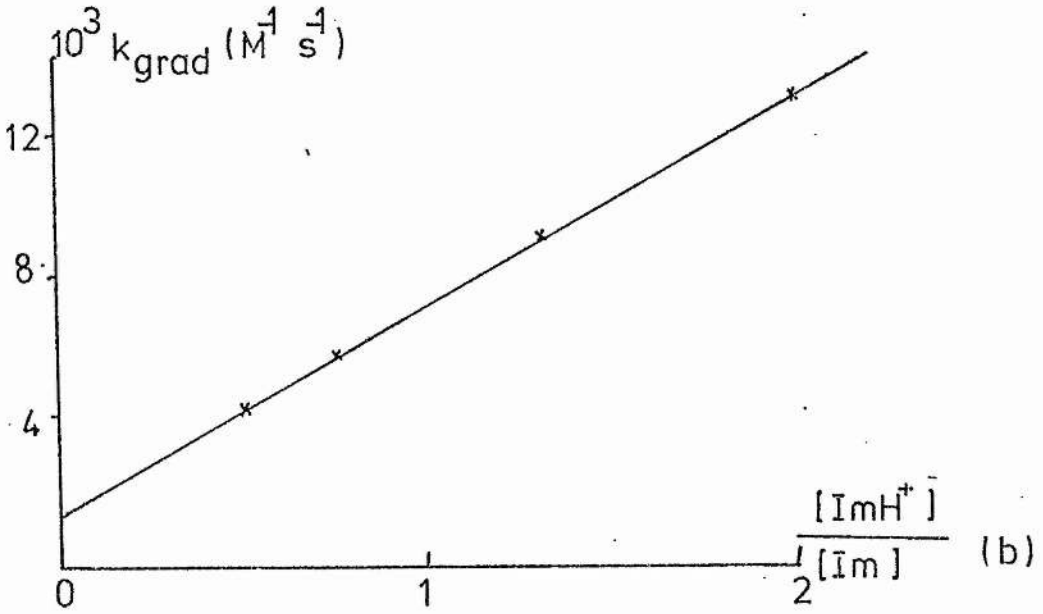


FIGURE 15. Reaction of benzylpenicillin with imidazole at 37°C.

B. IMIDAZOLE

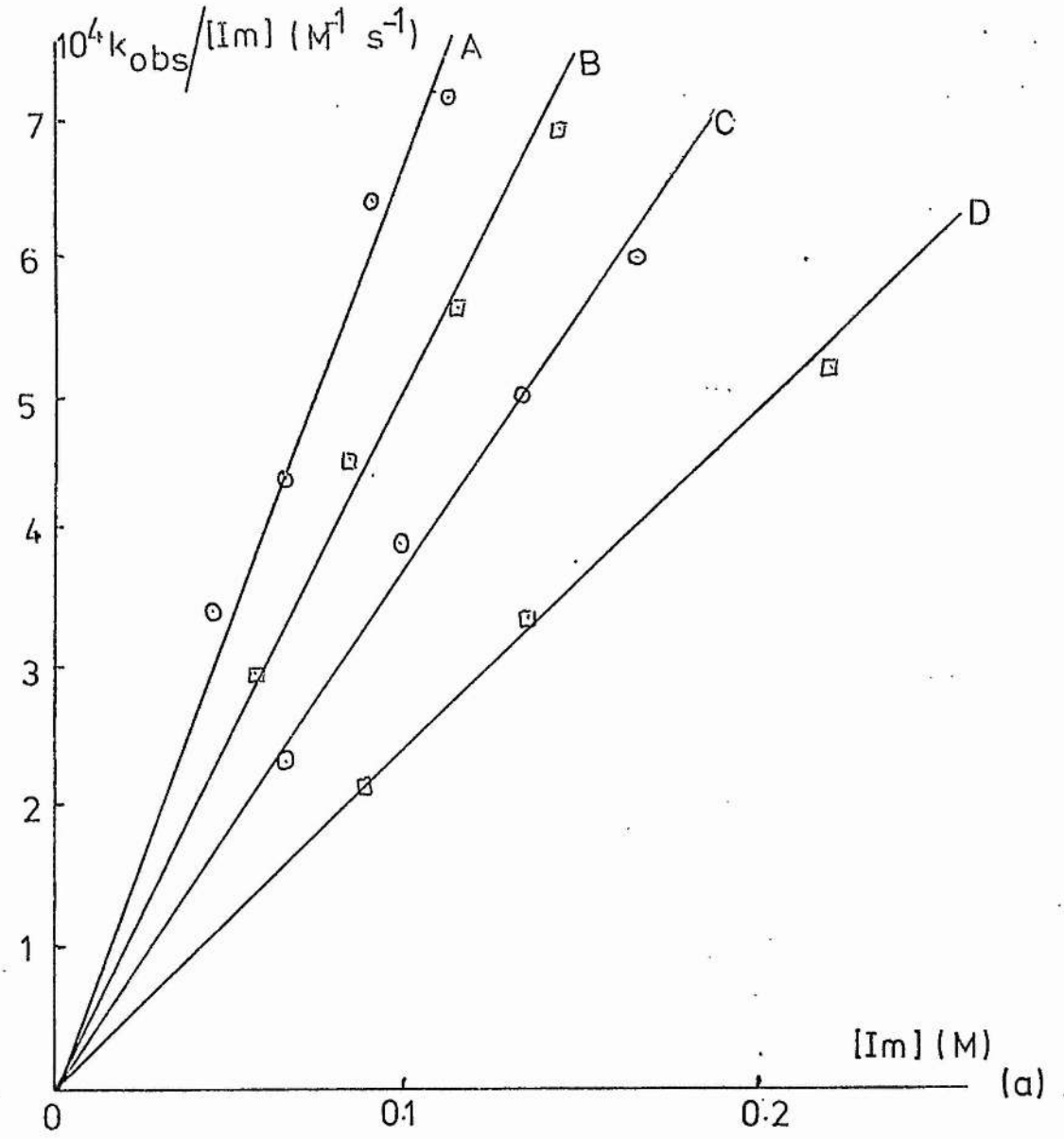
Table 30. Reaction of benzylpenicillin with imidazole at 37°C.

10^2 [Im]$_F$/M	$10^5 k_{obs}/s^{-1}$	10^2 [Im]$_F$/M	$10^5 k_{obs}/s^{-1}$
A - $[ImH^+]/[Im]$ = 2		B - $[ImH^+]/[Im]$ = 1.31	
pH = 6.66		pH = 6.84	
9.8	12.5	12.7	14.0
7.8	7.6	10.2	9.4
5.9	4.4	7.6	5.5
3.9	1.65	5.1	2.35
C - $[ImH^+]/[Im]$ = 0.76		D - $[ImH^+]/[Im]$ = 0.5	
pH = 7.07		pH = 7.26	
16.7	16.5	19.6	16.0
13.3	10.0	15.7	11.0
10.0	6.3	11.8	6.35
6.7	2.55	7.8	2.3

A plot of $k_{obs}/[Im]_F$ against $[Im]_F$ is shown in figure 15a. It can be seen that each set of points forms a straight line passing through the origin, the gradient of which is dependent upon the pH. At a fixed pH, then

$$k_{obs} = k_{grad}[Im]^2. \qquad - (iii)$$

The values of k_{grad} are summarised in table 31, and plotted in figure 15b against $[ImH^+]/[Im]$.



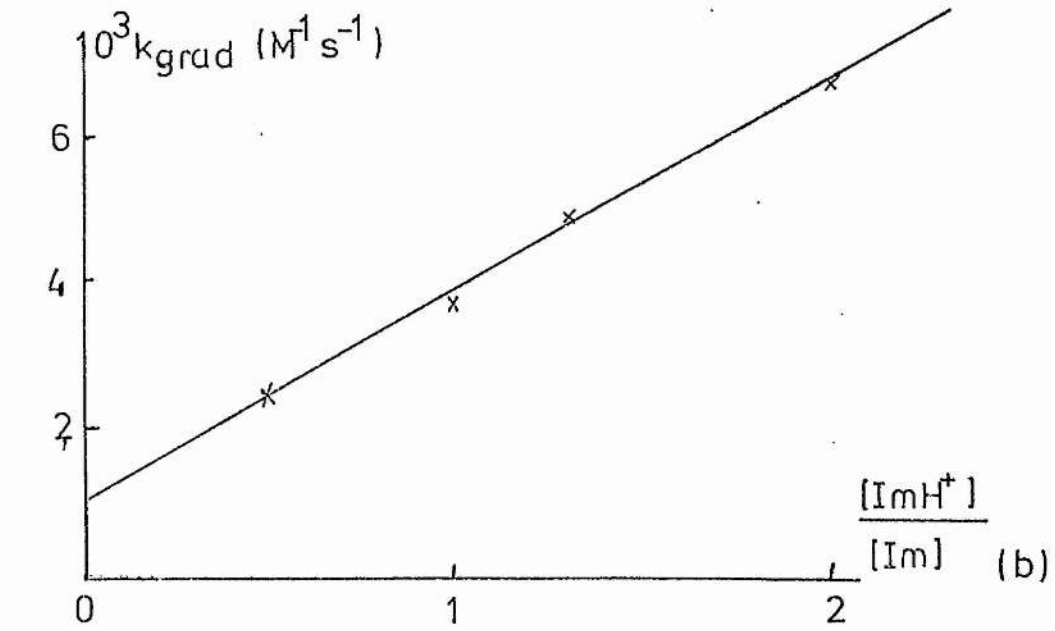


FIGURE 16. Reaction of benzylpenicillin with imidazole/D_2O at 37°C.

Table 31. Reaction of benzylpenicillin with imidazole at 37°C.

Solution	$[ImH^+]/[Im]$	$10^3 k_{grad}/M^{-2}s^{-1}$
A	2	12.7
B	1.31	9.1
C	0.76	5.8
D	0.50	4.4

Figure 15b shows a straight line with a non-zero intercept. This means that the overall rate equation is

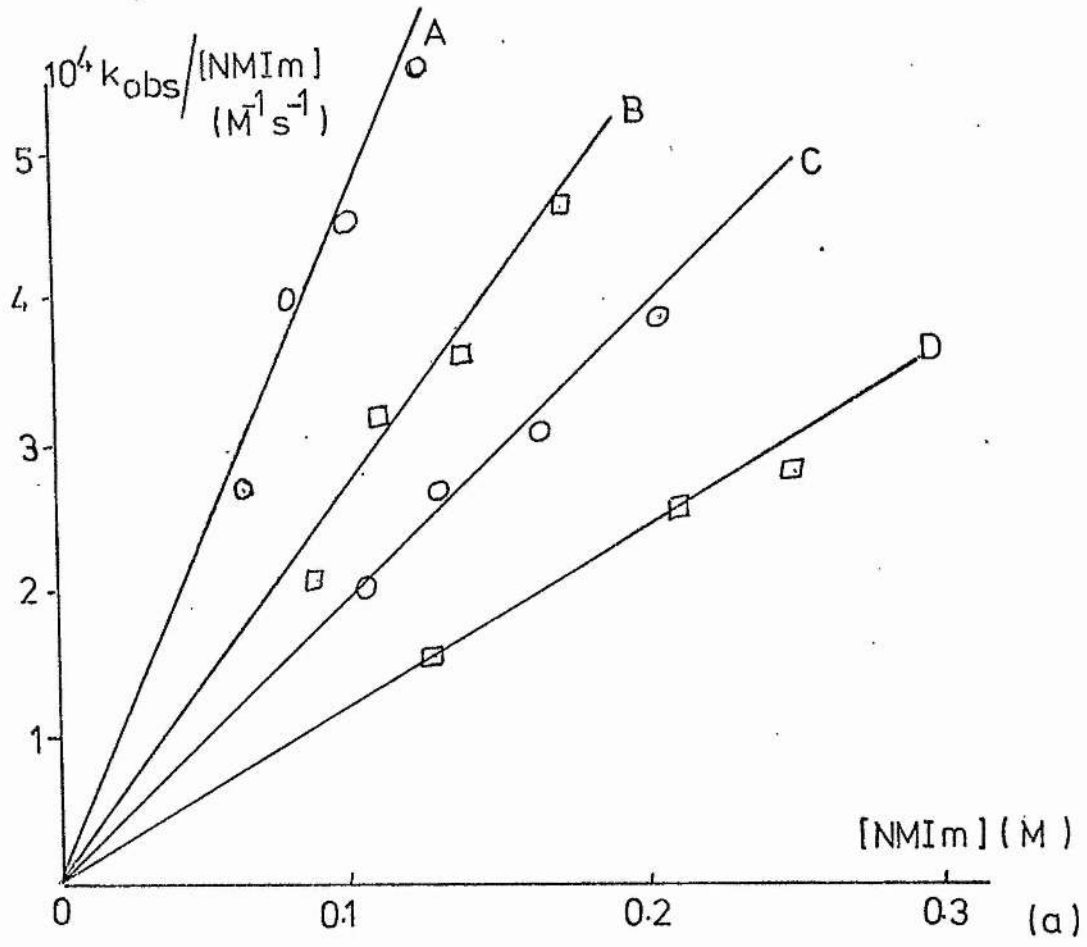
$$k_{obs} = k_1 [ImH^+][Im] + k_2 [Im]^2 \qquad - (i)$$

From measurements on the gradient and intercept of figure 15b, $k_1 = 5.8 \times 10^{-3}\ M^{-2}\ s^{-1}$ and $k_2 = 1.5 \times 10^{-3}\ M^{-2}\ s^{-1}$.

C. SOLVENT KINETIC ISOTOPE EFFECT (IMIDAZOLE)

Table 32. Reaction of benzylpenicillin with imidazole/D_2O at 37°C.

$10^2 [Im]/M$	$10^5 k_{obs}/s^{-1}$	$10^2 [Im]/M$	$10^5 k_{obs}/s^{-1}$
A - $[ImD^+]/[Im]$ = 2.01		B - $[ImD^+]/[Im]$ = 1.30	
11.4	8.2	14.5	10.0
9.1	5.7	11.6	6.45
6.8	3.0	8.7	3.9
4.6	1.5	5.8	1.7
C - $[ImD^+]/[Im]$ = 1.00		D - $[ImD^+]/[Im]$ = 0.49	
16.7	10.0	22.3	11.5
13.4	6.7	13.4	4.5
10.0	3.9	8.9	1.85
6.7	1.95		



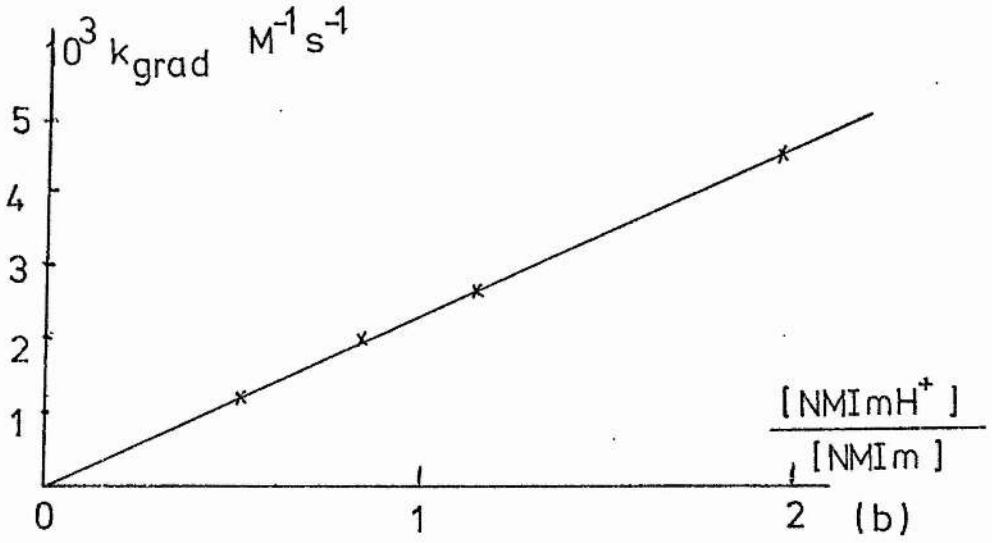


FIGURE 17. Reaction of benzylpenicillin with N-methylimidazole at 37°C.

These results are plotted in figure 16, and summarised in table 33.

Table 33. Reaction of benzylpenicillin with imidazole/D_2O at 37°C.

Solution	$[ImD^+]/[Im]$	$10^3 k_{grad}/M^{-2} s^{-1}$
A	2.01	6.7
B	1.30	4.8
C	1.00	3.7
D	0.49	2.4

Therefore, in the deuteriated buffer solutions, the rate constants, by inspection of figure 16b are: $k_1 = 2.9 \times 10^{-3}\ M^{-2}\ s^{-1}$ and $k_2 = 1.0 \times 10^{-3}\ M^{-2}\ s^{-1}$.

D. N-METHYLIMIDAZOLE

Table 34. Reaction of benzylpenicillin with N-methylimidazole at 37°C.

10^2 [NMIm]/M	$10^5 k_{obs}/s^{-1}$	10^2 [NMIm]/M	$10^5 k_{obs}/s^{-1}$
A - $[NMImH^+]/[NMIm]$ = 2.0		B - $[NMImH^+]/[NMIm]$ = 1.18	
pH = 6.80		pH = 7.03	
12.5	7.0	17.2	7.85
10.0	4.5	13.8	5.05
8.0	3.15	11.0	3.5
6.4	1.75	8.8	1.85
C - $[NMImH^+]/[NMIm]$ = 0.85		D - $[NMIMmH^+]/[NMIm]$ = 0.5	
pH = 7.18		pH = 7.41	
20.4	7.7	31.4	9.5
16.3	5.0	25.1	7.0
13.0	3.5	20.1	5.0
10.4	1.98	12.8	1.9

Once again, it was found that a plot of $k_{obs}/[NMIm]$ against $[NMIm]$ at constant pH gave a straight line passing through the origin (figure 17). (However, experiments performed with higher concentrations of N-methylimidazole gave points which fell below the appropriate line.) Thus, equation (iii) holds true for N-methylimidazole. The values of k_{grad} are recorded in table 35.

Table 35. Reaction of benzylpenicillin with N-methylimidazole at 37°C.

Solution	$[NMImH^+]/[NMIm]$	$10^3\ k_{grad}/M^{-2}\ s^{-1}$
A	2.0	4.55
B	1.18	2.7
C	0.85	2.0
D	0.5	1.2

The straight line in figure 17b this time passes through the origin. Thus the overall rate equation of N-methylimidazole is

$$k_{obs} = k_1\ [NMIm]\ [NMImH^+] \qquad \text{- (iv).}$$

From figure 17b, $k_1 = 2.3 \times 10^{-3}\ M^{-2}\ s^{-1}$.

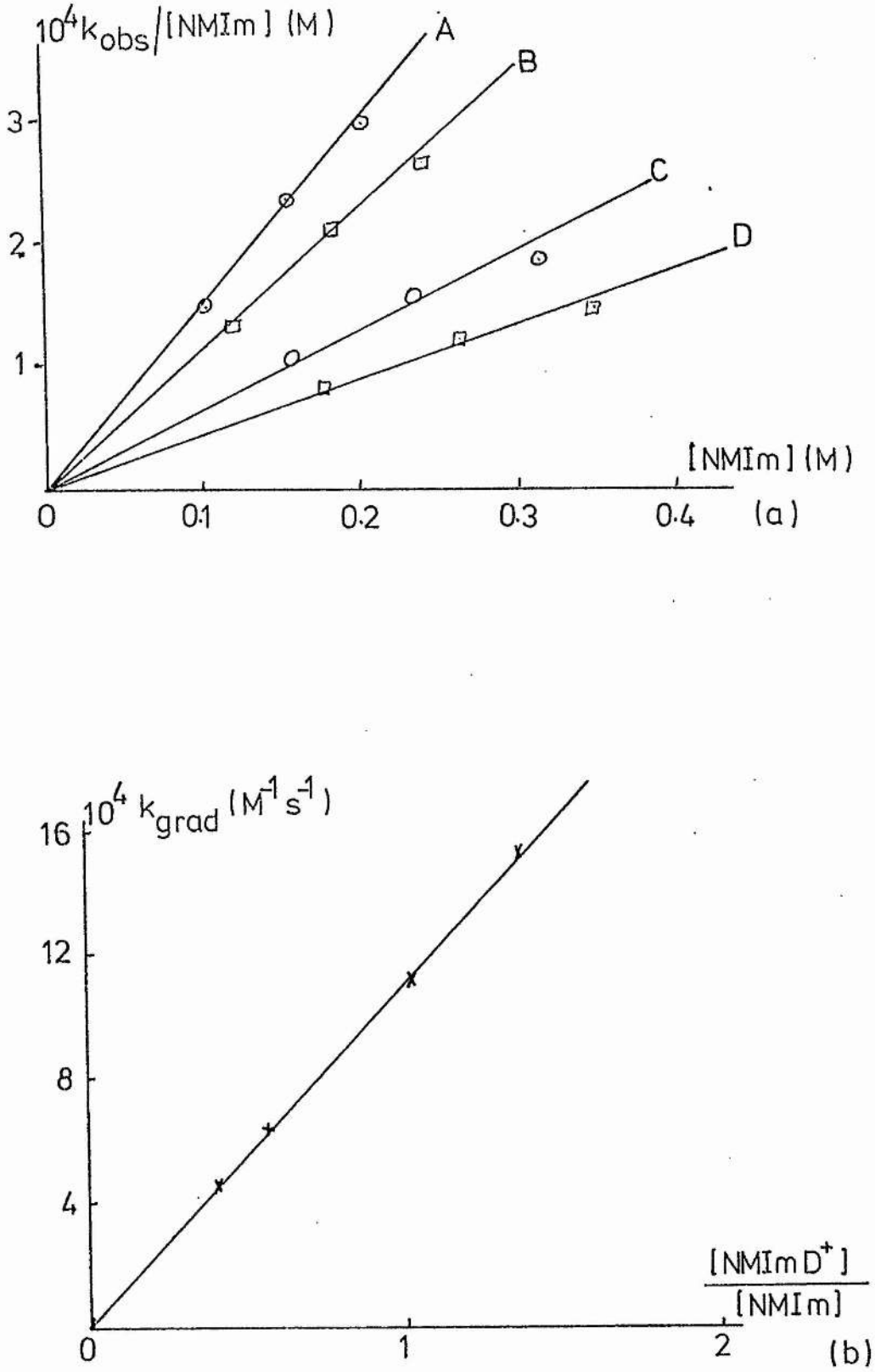


FIGURE 18. Reaction of benzylpenicillin with N-methylimidazole/D_2O at 37°C.

E. SOLVENT KINETIC ISOTOPE EFFECT (N-METHYLIMIDAZOLE)

Table 36. Reaction of benzylpenicillin with N-methylimidazole/D_2O at 37°C.

10^2 [NMIm] /M	$10^5 k_{obs}/s^{-1}$	10^2 [NMIm] /M	$10^5 k_{obs}/s^{-1}$
A - [$NMImD^+$] / [NMIm] = 1.38		B - [$NMImD^+$] / [NMIm] = 1.03	
26.0	8.0	30.5	8.25
20.8	6.15	24.4	6.4
15.6	3.65	18.3	3.75
10.4	1.45	12.2	1.6
C - [$NMImD^+$] / [NMIm] = 0.57		D - [$NMImD^+$] / [NMIm] = 0.41	
39.5	8.0	44.0	7.0
31.6	5.8	35.2	5.1
23.7	3.65	26.4	3.15
15.8	1.65	17.6	1.45

These results are plotted in figure 18, and the values of k_{grad} are summerised in table 37.

Table 37. Reaction of benzylpenicillin with N-methylimidazole/D_2O at 37°C.

Solution	[$NMImD^+$] / [NMIm]	$10^4 k_{grad}/M^{-2} s^{-1}$
A	1.38	15
B	1.03	11
C	0.57	6.1
D	0.41	4.3

From inspection of figure 18b, k_1 in the deuteriated buffers is 1.08×10^{-3} M^{-2} s^{-1}.

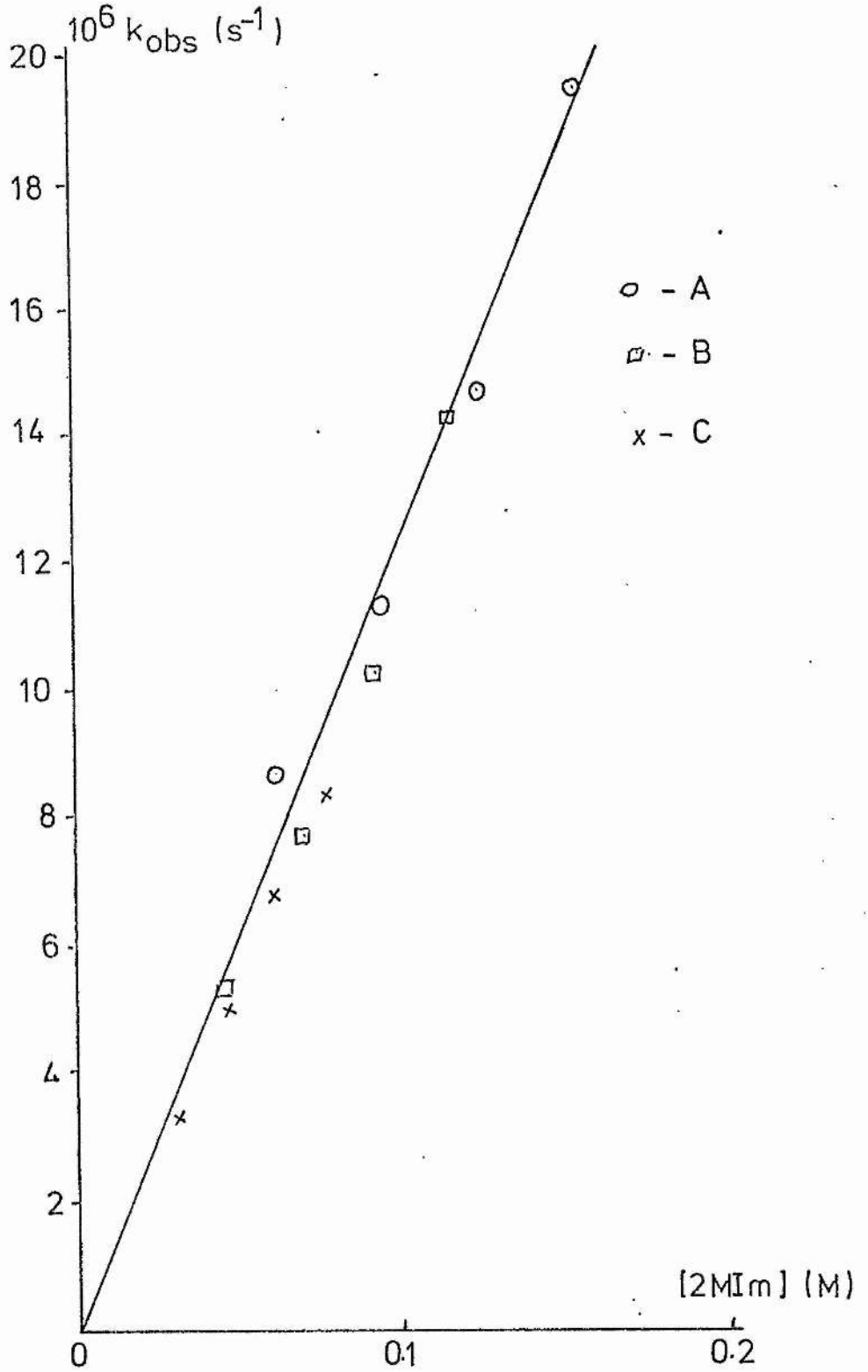


FIGURE 19. Reaction of benzylpenicillin with 2-methylimidazole at 37°C.

F. 2-METHYLIMIDAZOLE

Table 38. Reaction of benzylpenicillin with 2-methylimidazole at 37°C.

10^2 [2MIm]/M	10^6 k_{obs}/s^{-1}	10^2 [2MIm]/M	10^6 k_{obs}/s^{-1}
A - $[2MImH^+]/[2MIm]$ = 0.75		B - $[2MImH^+]/[2MIm]$ = 1.35	
pH = 8.01		pH = 7.75	
15.8	19.5	11.8	14.2
12.6	14.7	9.4	10.3
9.5	11.3	7.1	7.7
6.3	8.7	4.7	5.3

C - $[2MImH^+]/[2MIm]$ = 2.55

pH = 7.48

7.8	8.3
6.2	6.8
4.7	5.0
3.1	3.3

It is found in this case that a straight line is obtained when k_{obs} is plotted against [2MIm], and that the same line is obtained regardless of the pH. This line passes through the origin. It is shown in figure 19. (Again, if the experiments are performed at concentrations of 2-methylimidazole higher than this, the points do not coincide with the line.)

Therefore, the rate equation for 2-methylimidazole is

$$k_{obs} = k_3 [2MIm] \qquad - (v).$$

By inspection of figure 19, $k_3 = 1.2 \times 10^{-4}\ M^{-1}\ s^{-1}$.

G. SOLVENT KINETIC ISOTOPE EFFECT (2-METHYLIMIDAZOLE)

Table 39. Reaction of benzylpenicillin with 2-methylimidazole/D_2O at 37°C.

$[2MImD^+] / [2MIm] = 1.60$

10^2 [2MIm] /M	17.3	14.4	11.5
10^6 k_{obs}/s^{-1}	11.0	8.9	7.2

Again, a straight line is obtained when k_{obs} is plotted against [2MIm]. Its gradient is 6.2×10^{-5} M^{-1} s^{-1}.

H SUMMARY

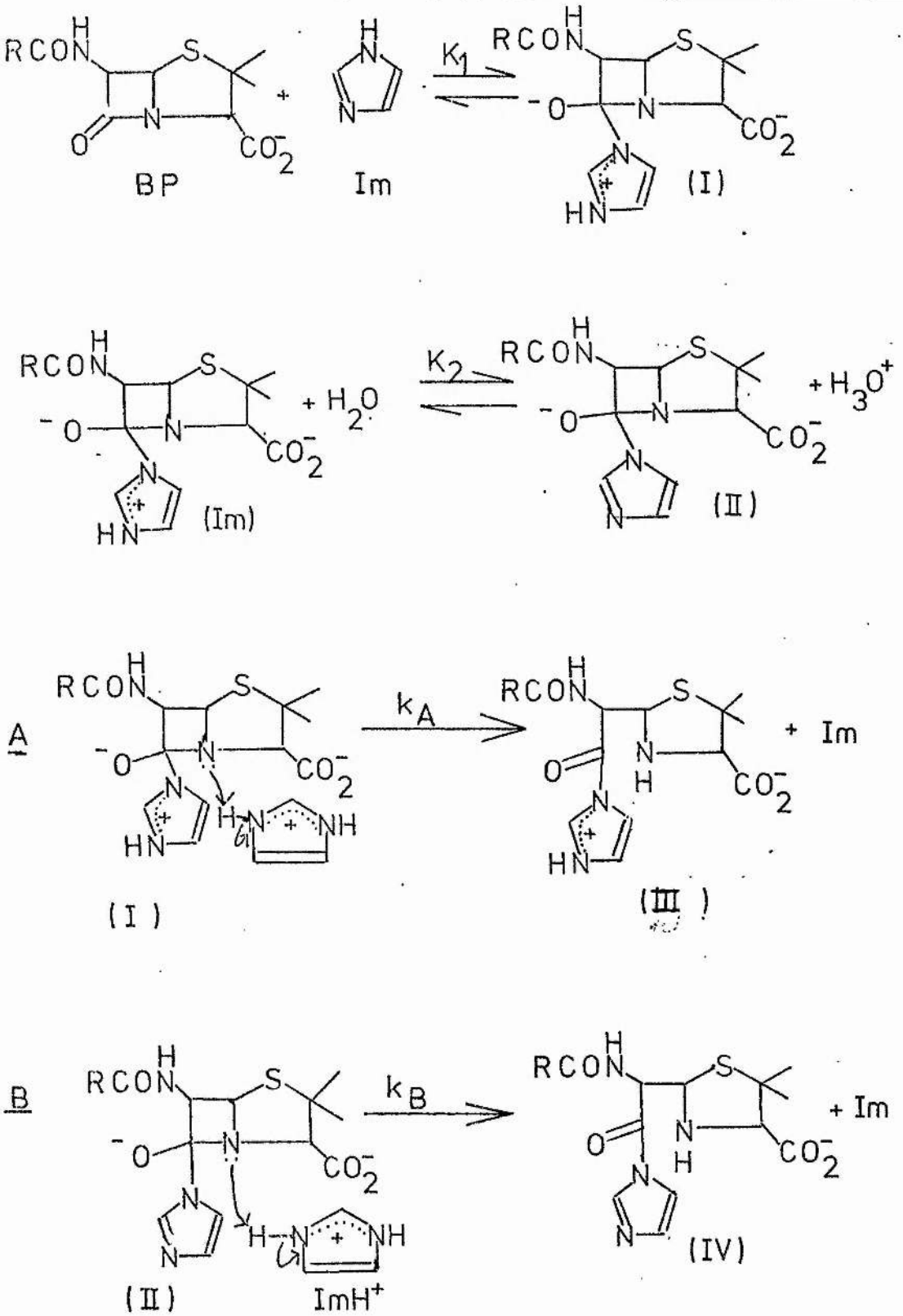
In general, the rate equation is

$$k_{obs} = k_1 [Im] [ImH^+] + k_2 [Im]^2 + k_3 [Im]$$

The values for these rate constants are summarised in table 40.

Table 40. Reaction of benzylpenicillin with substituted imidazoles at 37°C.

	10^3 k_1/M^{-2} s^{-1}	10^3 k_2/M^{-2} s^{-1}	10^4 k_3/M^{-1} s^{-1}
Imidazole (H_2O)	5.8	1.5	-
Imidazole (D_2O)	2.9	1.0	-
N-methylimidazole (H_2O)	2.3	-	-
N-methylimidazole (D_2O)	1.08	-	-
2-methylimidazole (H_2O)	-	-	1.2
2-methylimidazole (D_2O)	-	-	0.62



SCHEME 25

DISCUSSION

On the basis of the foregoing results, the mechanism outlined in scheme 25 is suggested to account for step A in Bundgaard's proposed scheme (scheme 24).

It is envisaged that, as a first step, the tetrahedral intermediate (I) is formed in equilibrium with penicillin and imidazole. The equilibrium constant K_1 is given by

$$K_1 = \frac{[(I)]}{[BP][Im]} \qquad - (vi)$$

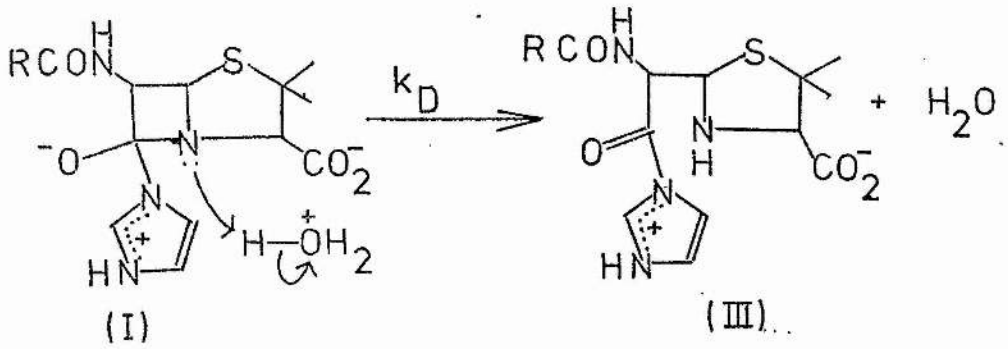
Once formed, (I) comes into equilibrium with its deprotonated form (II). The equilibrium constant for this, K_2, is given by

$$K_2 = \frac{[(II)][H_3O^+]}{[(I)][H_2O]} \qquad - (vii)$$

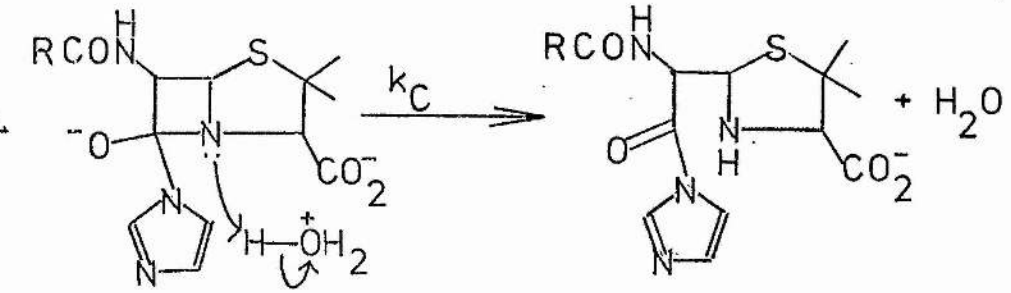
The value of K_2 would be expected to be similar to that for any N-substituted imidazole; ie roughly 2×10^{-9}. This equilibrium is impossible in the case of N-methylimidazole.

The rate-determining step is thought to be proton transfer to the tetrahedral intermediate (either (I) or (II)), leading to immediate cleavage of the lactam ring and formation of intermediates (III) and (IV). The protonating agent can be either H_3O^+ or ImH^+. Thus four rate limiting steps can be envisaged, giving up to four terms in the rate equation. These steps are:

D



C



SCHEME 25 (contd)

A. Proton transfer from ImH^+ to (I).

$$\text{rate} = k_A [(I)] [ImH^+] \quad \text{- (viii)}$$

$$= k_1 [Im] [ImH^+] [BP] \quad \text{- (ix)}$$

$$\text{where} \quad k_1 = k_A K_1 \quad \text{- (x)}$$

This step gives rise to the k_1 term observed in the experiments.

B. Proton transfer from ImH^+ to (II).

$$\text{rate} = k_B [ImH^+] [(II)] \quad \text{- (xi)}$$

$$= k_2 [Im]^2 [BP] \quad \text{- (xii)}$$

$$\text{where} \quad k_2 = k_B K_1 K_2 [H_2O] / K_a \quad \text{- (xiii)}$$

$$K_a = [Im] [H^+] / [ImH^+] \quad \text{- (xiv)}$$

K_a has a value of roughly 10^{-7} M.

C. Proton transfer from H_3O^+ to (II)

$$\text{rate} = k_C [(II)] [H_3O^+] \quad \text{- (xv)}$$

$$= k_3 [Im] [BP] \quad \text{- (xvi)}$$

$$\text{where} \quad k_3 = k_C K_1 K_2 [H_2O] \quad \text{- (xvii)}$$

D. Proton transfer from H_3O^+ to (I).

$$\text{rate} = k_D [(I)] [H_3O^+] \quad \text{- (xviii)}$$

$$= k_4 [ImH^+] [BP] \quad \text{- (xix)}$$

$$\text{where} \quad k_4 = k_D K_1 K_a \quad \text{- (xx)}$$

Of these four terms, only those corresponding to steps A and B actually appear in the rate equation for imidazole. For N-methylimidazole, only steps A and D are possible; and in fact only step A is observed.

If all of these protonation steps took place at similar rates, it would be impossible to observe the k_3 and k_4 terms, because they would

be 10^7 times smaller than k_1 and k_2. However, Tsuji et al.[91] do claim to have detected a k_3-type term, with $k_3 = 9.7 \times 10^{-6}$ M^{-1} s^{-1}. It is doubtful if a rate constant which is so small in relation to the larger constants can be determined with any degree of accuracy; and even if it is correct, it is unlikely to arise from step C of the proposed mechamism. If it did, it would mean that protonation by H_3O^+ occurred ca. 10^4 times faster than protonation by ImH^+, and therefore a k_4-type term ought to be observed also. Further, the same authors report a k_3 -type term ($k_3 = 9.7 \times 10^{-6}$ M^{-1} s^{-1}) for the reaction of benzylpenicillin with N-methylimidazole, which cannot possibly take part in step C.

In the case of N-methylimidazole, k_1 has about half the value obtained for imidazole. This difference is not very significant; but it most probably reflects a slight steric hindrance by the methyl group of the proton transfer step.

The isotope effects (k_{H_2O}/k_{D_2O}) are 2.0 for the k_1 term and 1.5 for the k_2 term. Unfortunately, nothing definite can be deduced from these values. The best that can be said is that they are consistent with the proposed mechanism. k_1 is the product of k_A and K_1. K_1 ought not to be at all affected by the change of isotope, and k_A would be expected to be considerably reduced in D_2O, since deuteron transfer is normally slower than proton transfer. The isotope effect in thus much smaller than expected; but this could be due to many complicated factors, and it does not by itself disprove the mechanism.

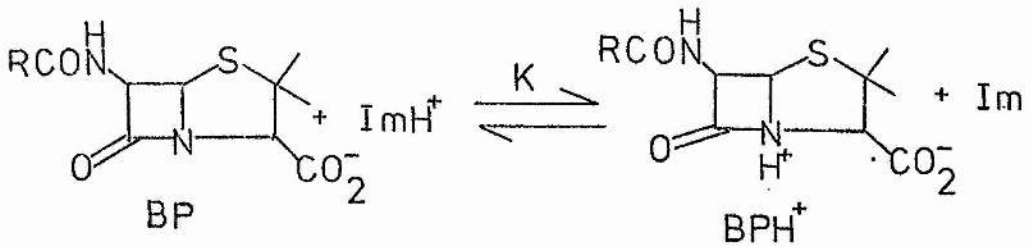
In the case of 2-methylimidazole, only a k_3 term is observed. This term, however, is unlikely to arise from step C of the proposed mechanism. Its value is an order of magnitude greater than Tsuji's estimate for this term with imidazole[91]. This in itself is not

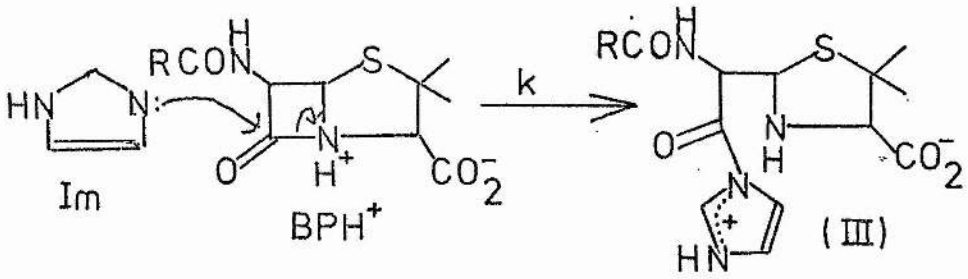
unreasonable, from a consideration of the pK_a values. However, if step C does occur with 2-methylimidazole, it is hard to see why steps A and B do not occur also. It may be urged that steps A and B do not operate with 2-methylimidazole because it is a stronger base than imidazole and therefore proton transfer from the conjugate acid is less favourable. However, it seems unlikely that the steps would cease altogether, when the difference in pK_a values is only 1 unit.

The observed rate expression may be explained by an alternative mechanism. One possibility would be direct rearrangement of (I) to (III) by an intramolecular proton transfer. However, if this were the case, this process should also occur with the other imidazoles, at the same time as the intermolecular proton transfer. There is, then, no reason why the two processes should not operate simultaneously with 2-methylimidazole.

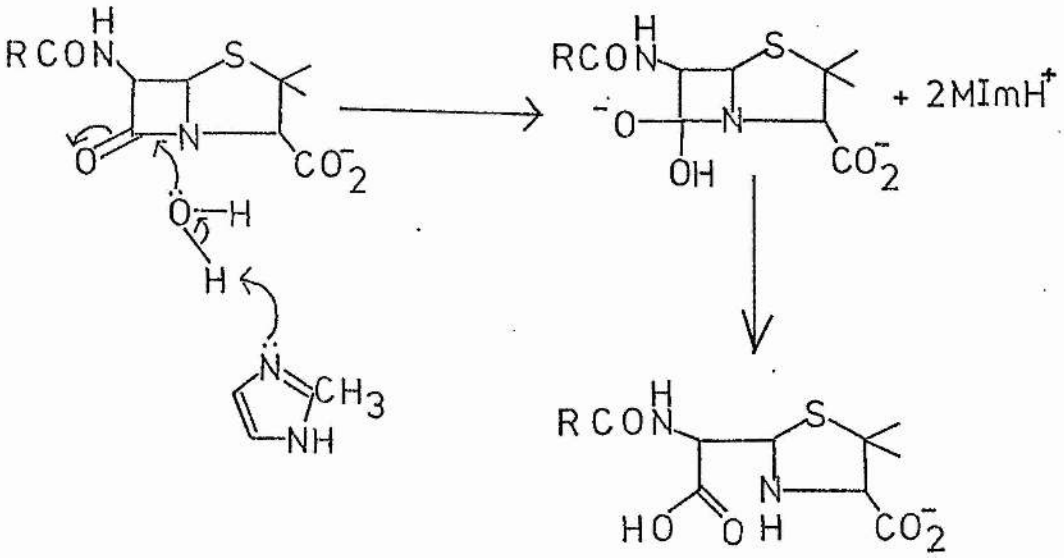
The most likely explanation of this rate expression is that, with 2-methylimidazole, formation of the tetrahedral intermediate (I) becomes the rate-determining step. The relative slowness of this step, compared with the analogous step in the imidazole and N-methylimidazole reactions, may be attributed to hindrance by the methyl group ortho to the reactive centre of the molecule. This effect would not be so significant if the methyl group were meta to the reacting nitrogen, as in N-methylimidazole. Thus, although a consideration of pK_a values would suggest that 2-methylimidazole is the best nucleophile of the three, steric effects considerably reduce its activity. It is well known[96] that 2-methyl-imidazole is a much poorer nucleophile than imidazole.

The higher basicity of 2-methylimidazole, combined with its poor nucleophilicity, can also explain why the percentage conversion of penicillin to penicillenic acid is lower with this catalyst. The mechanism





SCHEME 27



SCHEME 26

proposed is a type of nucleophilic catalysis. But 2-methylimidazole can also act as a general base catalyst - catalysing by a different mechanism (scheme 26). In the imidazole-catalysed hydrolysis of esters, both these types of catalysis have been detected: however, both lead to the same products. In the present reaction, though, general base catalysis does not lead to formation of penicillenic acid, but rather of penicilloic acid.

For N-methylimidazole and imidazole, the proposed mechanism satisfactorily accounts for the observed rate law. Further, it can be demonstrated that no other mechanism fits the observed behaviour. One alternative mechanism is illustrated in scheme 27. This envisages that the rate-determining step is nucleophilic attack of imidazole on protonated penicillin, leading to (III): the penicillin having previously been protonated in an equilibrium process by imidazolium ion. This scheme incorporates the same steps as scheme 25, but it would not give rise to the same kinetics. For this process, it can be shown that

$$\text{rate} = kK [ImH^+] [BP] \qquad - (xxi)$$

Yet another suggestion might be that the proton transfer steps are themselves equilibrium processes, and that the rate-determining step is the spontaneous explusion of imidazole from (III) (step B in scheme 24). This, however, would also produce kinetics of the form

$$\text{rate} = k [ImH^+] [BP] \qquad - (xxii)$$

That this step is not rate-determining is also demonstrated by the work of Bundgaard[36]. He finds that the rates of the imidazole -

catalysed reactions of a range of penicillins can be correlated with their rates of alkaline hydrolysis. If the intramolecular nucleophilic displacement on (III) were rate-determining, then the rates would be expected to correlate with those of the acid-catalysed reactions. For example, the side-chain carbonyl group in penicillin V is considerably less nucleophilic than in penicillin G: yet the imidazole-catalysed reaction of penicillin V occurs some 25% faster.

In summary, it can be deduced by a process of elimination that the proton transfer must be rate-determining. One would normally expect such a step to have a very low activation energy; it follows that it should be diffusion-controlled. Now, it is generally accepted that diffusion-controlled bimolecular reactions have rate constants of the order of 10^{10} M^{-1} s^{-1} [97]. For the present case, this means that the third-order rate constants should be of the order of $10^{10}K_1$.

Gensmantel and Page[46] have estimated equilibrium constants for reactions between benzylpenicillin and a number of amines, leading to tetrahedral intermediates analogous to (I). They were able to correlate these with the pK_a values for the amines. On the basis of their data, K_1 for imidazole should be in the region of 4×10^{-12} M^{-1}. This suggests that the rate constants should be in the region of 4×10^{-2} M^{-1} s^{-1}. That they are in fact an order of magnitude less than this may indicate that, for this reaction, there is a higher than usual barrier to proton transfer.

Proton transfer to the negatively charged oxygen of (I) or (II) is, of course, a more favoured process, but this would be non-productive. Once the nitrogen has been protonated the ensuing steps are irreversible.

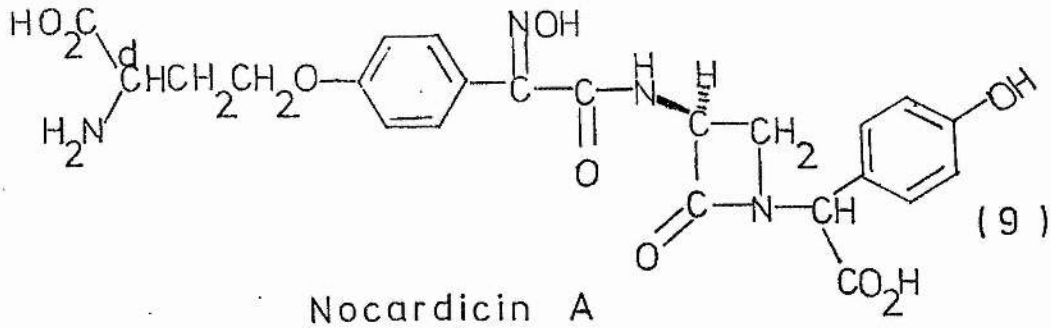
Morris and Page[98] have discussed, in detail, the role of proton transfer in the aminolysis of benzylpenicillin, and the mechanism

proposed here is consistent with the criteria they established.

The foregoing analysis has demonstrated that it is not necessary to postulate general base catalysis of the type shown in scheme 24 (where one imidazole molecule enhances the nucleophilicity of a second one) in order to explain the rate equation. The term in $[\,Im\,]^2$ arises from the algebra of the reaction rather than the chemistry.

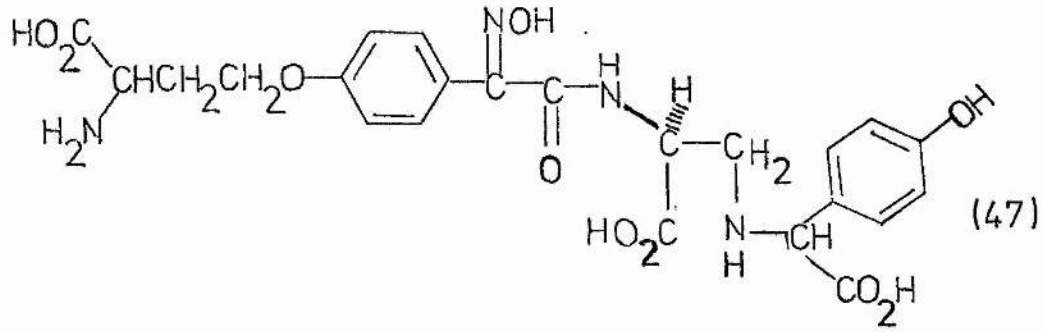
CHAPTER FIVE

A KINETIC STUDY OF THE ACID HYDROLYSIS OF NOCARDICIN A



(9)

Nocardicin A



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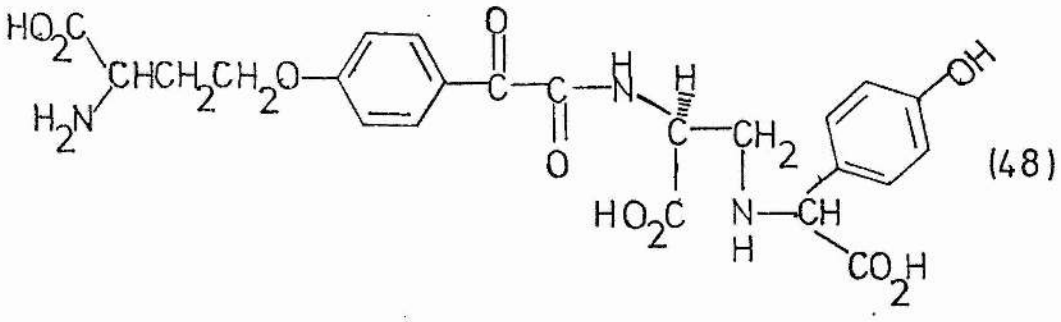

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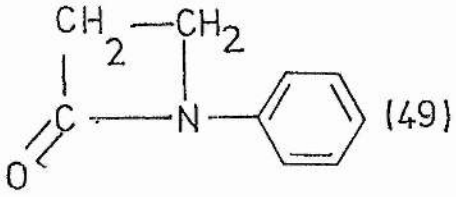

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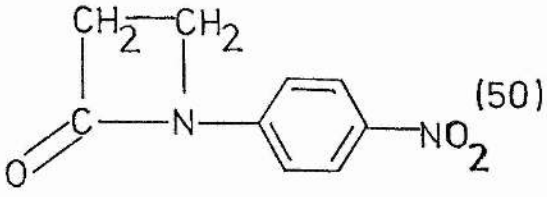


(50)

INTRODUCTION

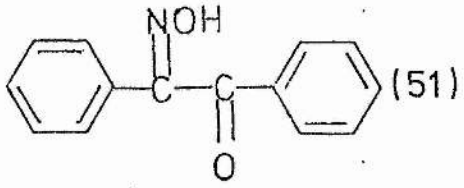
It was for many years supposed that the β-lactam antibiotics owed their antibacterial activity, at least partly, to the fact that the lactam ring is fused to another small ring. This is the case with the β-lactams discovered first: the penicillins and cephalosporins. Also, it is well-known that simple mono-cyclic β-lactam compounds are remarkably unreactive chemicals. (As an example, in the reaction of N-phenyl-azetidin-2-one (49) with hydroxide ion, the second order rate constant at 30°C is 2.5×10^{-3} M^{-1} s^{-1} [99]. This may be compared with 150×10^{-3} M^{-1} s^{-1} for benzylpenicillin.)

Recently, however some mono-cyclic β-lactam antibiotics have been discovered. Among these are nocardicin A (9), isolated as a major component from the fermentation broth of a subspecies of Nocardia uniforms[100]. This substance acts exclusively against gram-negative bacteria, on which it has an inhibitory effect on cell-wall-synthesis.

The action of alkali on nocardicin A leads to β-lactam cleavage and to compound (47). The action of acid leads to compound (48) in which the oxime function as well as the β-lactam function has been hydrolysed[27].

It seemed a valuable exercise to investigate the kinetics of these reactions, and to compare the results with those for penicillins, simple mono-cyclic β-lactams, and simple oximes.

The β-lactams chosen for study were N-phenylazetidin-2-one (49) and N-p-nitrophenylazetidin-2-one (50). Since the technique used for this study is ultraviolet spectroscopy, it is necessary to have aryl groups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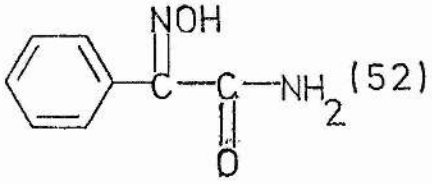
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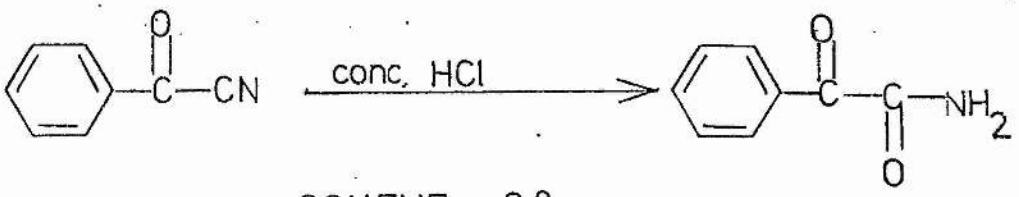
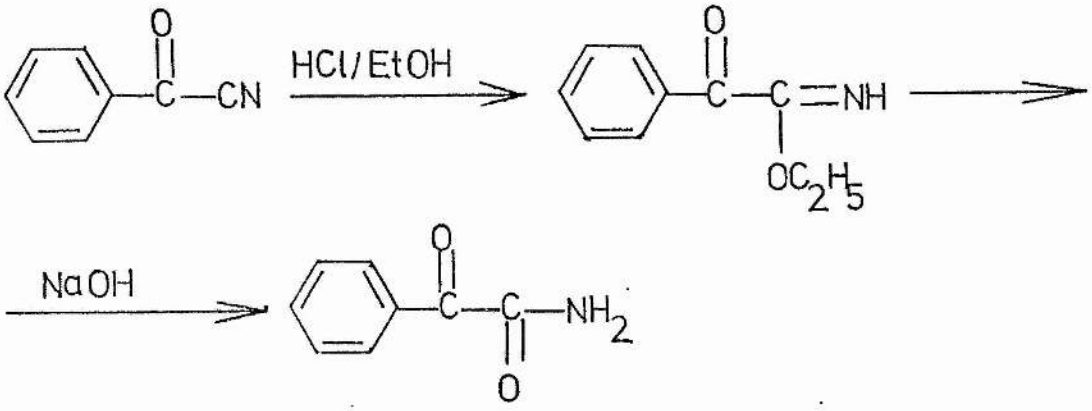
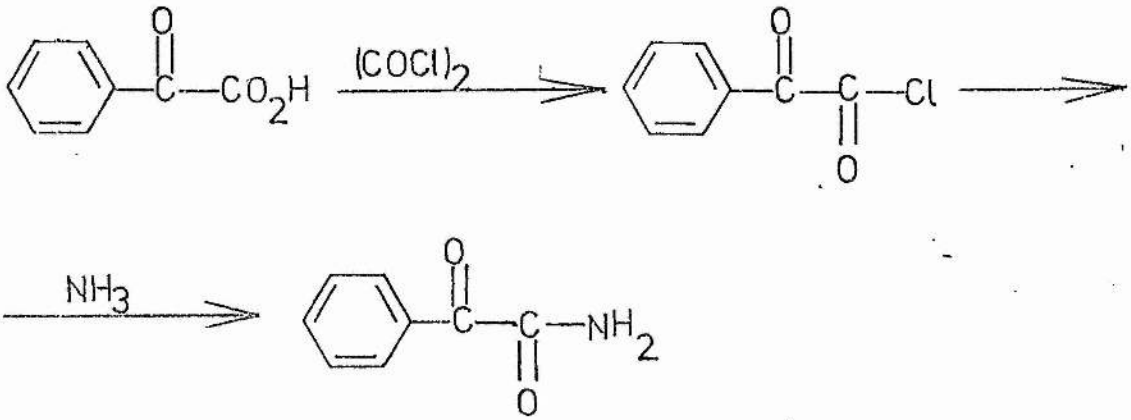


SCHEME 28



SCHEME 29



SCHEME 30

directly attached to nitrogen; otherwise no spectral changes can be observed.

The oximes were chosen so as to resemble most closely the oxime part of the nocardicin A molecule. First, benzil mono-oxime (51) was studied; then, when this proved unsatisfactory, benzoyl formamide mono-oxime (52).

The synthesis of this latter compound presented a few problems. Claisen[101] has described the preparation of benzoyl formamide by the action of concentrated hydrochloric acid on benzoyl cyanide (scheme 28). On following his procedure, however, it was found that the results could not be repeated. Indeed the product appeared to be benzoyl formic acid. Substitution of concentrated sulphuric acid for hydrochloric acid gave benzoic acid as the major product. An attempt to form the amide from the cyanide via the intermediacy of an imino-ether (scheme 29) also resulted in the formation of benzoic acid. Finally, the amide was prepared in a standard way from benzoyl formic acid via benzoyl formyl chloride (scheme 30).

EXPERIMENTAL

MATERIALS

Nocardicin A sodium salt was kindly supplied by its manufacturers: the Fujisawa Pharmaceutical Company of Osaka, Japan.

N-phenylazetidin-2-one and N-p-nitrophenylazetidin-2-one were prepared by Freeman in the course of his PhD research[102].

Benzil mono-oxime was prepared as follows[103]. Hydroxyammonium chloride (2g) was dissolved in a little water, and an equivalent amount of

sodium hydroxide (1.15g) was added. This concentrated aqueous solution of hydroxylamine was added to a saturated ethanolic solution of benzil (2.5g). The sodium chloride which precipitated on mixing was filtered off immediately, and the resulting solution was left to stand at room temperature for six days. The ethanol was evaporated off, leaving a yellow oily substance which crystallised on standing. The crystals were dissolved in a little ethanol, and on addition of a large volume of water a white precipitate slowly settled out. This was filtered off, washed with water and recrystallised from ethanol/water. It was dried in vacuo at 80°C. (Yield 0.5g, 19%); M.p. 95 - 105°C. (Found C, 74.5; H, 4.94; N, 6.03. $C_{14}H_{11}NO_2$ requires C, 74.65; H, 4.92; N, 6.22%.) (The lack of a sharp melting point can be attributed to the fact that the sample is a mixture of the α and β forms of the oxime.)

Benzoyl formic acid was prepared by the method of Oakwood and Weisgerber[104]. It was converted to benzoyl formyl chloride as described by Kharasch and Brown[105]. Thus benzoyl formic acid (15g) was dissolved in oxalyl chloride (35 ml). The solution was heated under reflux on a steam bath for six hours in the absence of moisture. The excess of oxalyl chloride was removed on a dry rotary evaporator. Toluene (50 ml) was added, and it too was removed by evaporation. The liquid residue was distilled under vacuum, the product coming over as a yellow oil. B.p. 62°C/0.5 mm Hg, (lit., 91°C/9.5 mm Hg), (Yield 11g, 65%.) I.R.: ν max at 1770 cm^{-1}, 1730 cm^{-1}. (Found C, 57.1; H, 2.88; Cl, 21.0. $C_8H_5ClO_3$ requires C, 57.00; H, 2.99; Cl, 21.03%.)

Benzoyl formamide was prepared as follows. Concentrated ammonia (150 ml) was cooled in an ice-bath, and 11g of benzoyl formyl chloride was dropped in slowly. The reaction was immediate - a pale yellow precipitate was formed. This was filtered off, washed in dilute ammonia and dried.

(Yield 4.7g 48%). Mp 125°C, (lit. 129°C[106]). IR: ν_{max} 3424 cm^{-1}, 1720 cm^{-1} 1685 cm^{-1}. (Found C, 64.1; H, 4.70; N, 9.5. $C_8H_7NO_2$ requires C, 64.42; H, 4.73; N, 9.39%.)

Benzoyl formamide mono-oxime was prepared from benzoyl formamide in the manner previously described for benzil mono-oxime. (Yield 0.8g, 48%.) Mp 172°C. IR: ν_{max} at 3420 cm^{-1}, 3130 cm^{-1}, 1640 cm^{-1}. (Found C, 58.7; H, 5.01; N, 16.7. $C_8H_8N_2O_2$ requires C, 58.53; H, 4.91; N, 17.06%.)

a BUFFER SOLUTIONS

Solutions in the pH range 1 - 1.5 were prepared by mixtures of 0.2M HCl and 0.2M KCl. pH was measured as described in Chapter 3.

Solutions in the H_o range (-1) - (-3) were prepared by mixtures of sulphuric acid and water. Concentration was determined by measurement of the specific gravity of each solution and the H_o values were obtained from tables[107]. The results are given in table 41.

Table 41. H_o values for sulphuric acid solutions

H_2SO_4 solution	Spec. Grav.	%Acid w/w	$-H_o$
A	1.3501	45.5	3.00
B	1.3397	44.0	2.86
C	1.3232	42.5	2.73
D	1.3067	40.5	2.58
E	1.2816	37.5	2.37
F	1.2712	36.5	2.30
G	1.2545	34.5	2.15
H	1.2364	32.0	1.96
I	1.2163	30.0	1.82
J	1.1964	27.0	1.61

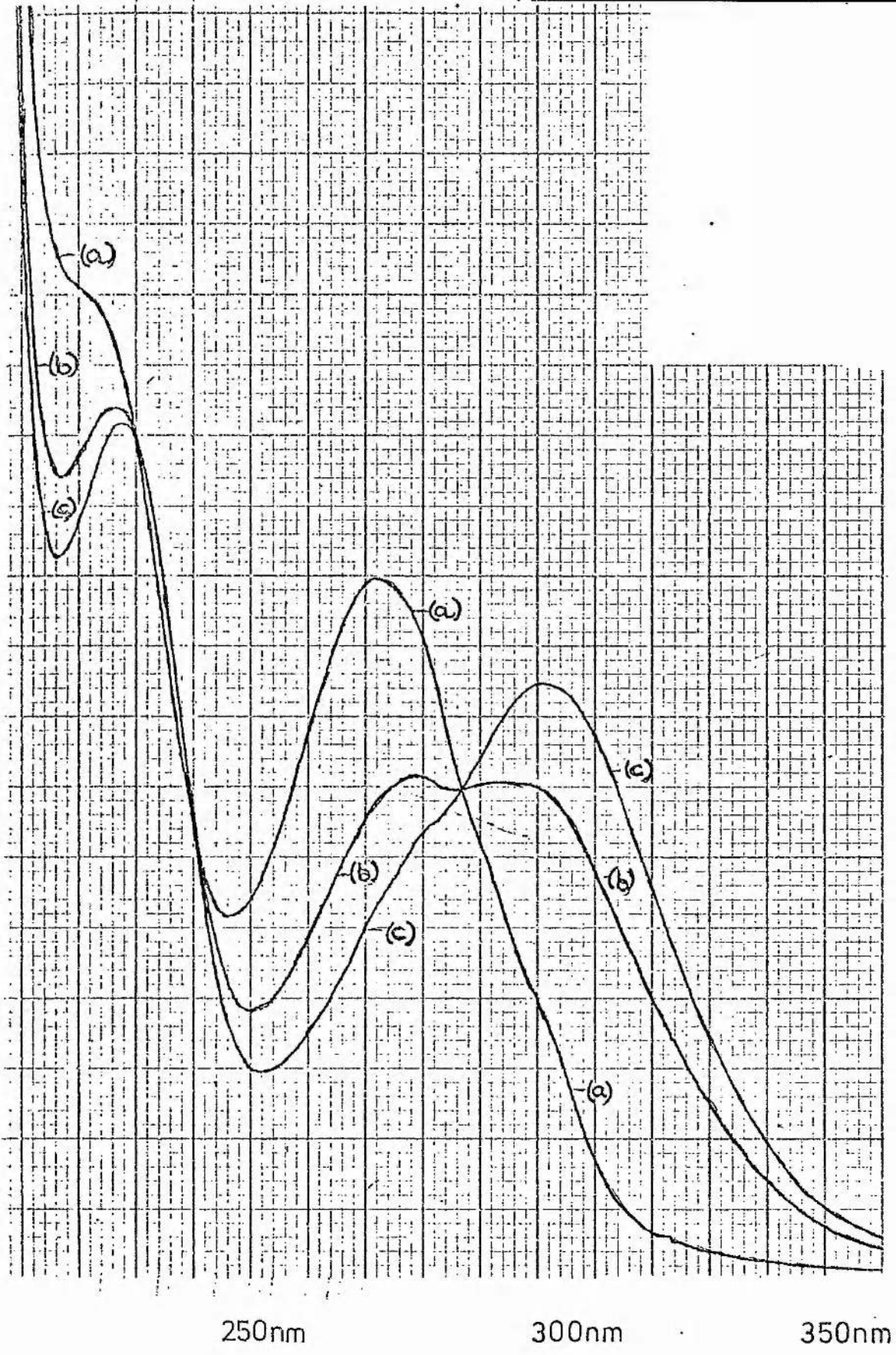


FIGURE 21. UV spectrum of nocardicin A in 0·1M HCl. a) initial; b) intermediate; c) final (24 hours).

c PROCEDURE

Experiments in the pH range were performed at 37°C; those in moderately concentrated sulphuric acid were performed at 30°C. Ultraviolet absorbances were measured as described previously, and psuedo-first-order rate constants were obtained by analysing the data by the method of Swinbourne[76].

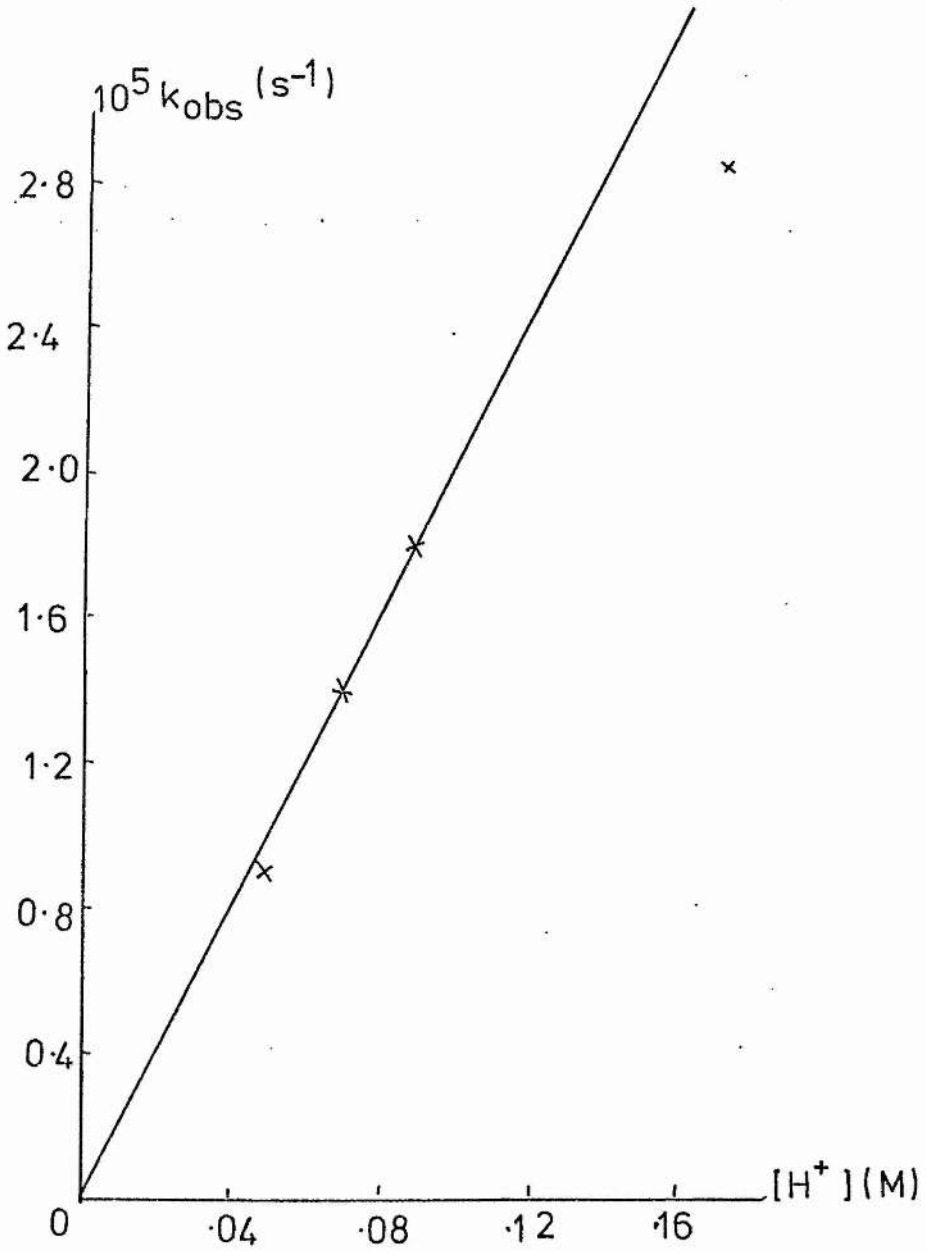
RESULTS

NOCARDICIN A

The ultraviolet spectrum of nocardicin A was taken in aqueous solution, and is reproduced in figure 21 opposite (line a). λ_{max} is at 271 nm (ε_{271} = 14,600). The spectrum taken in 0.1M NaOH solution was identical with this, and did not alter during 24 hours. The spectrum taken in 0.1M HCl solution was also identical; but this spectrum was observed to change, so that after 48 hours it had altered to line c. Line b is an intermediate stage. The maximum shifted to 299 nm. Isosbestic points are observed at 286 nm, 240 nm and 228 nm.

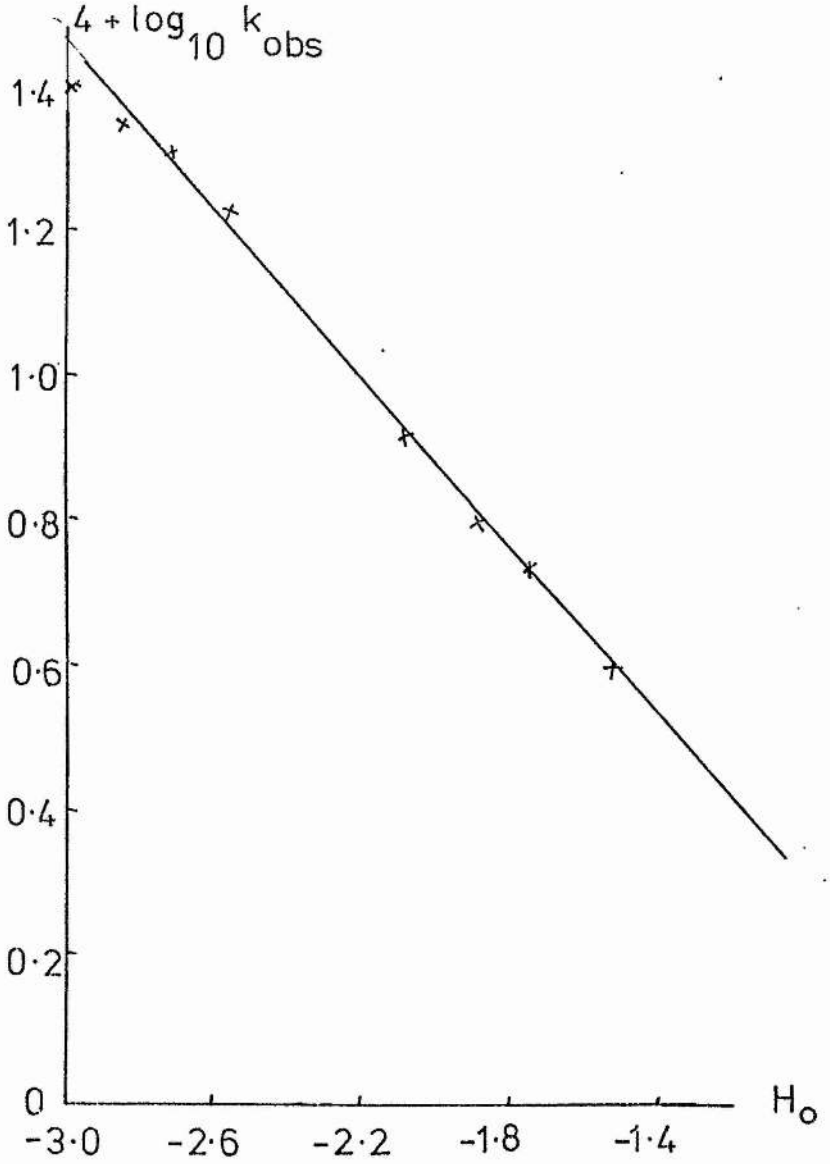
The kinetics of the reaction in acid (transformation of (9) to (48)) were initially determined in four buffer solutions in the pH range, by following the decrease of optical density at 270 nm. The concentration of nocardicin A in the buffers was 1.15×10^{-4} M.

Figure 22 shows a plot of k_{obs} against $[H^+]$. There appears to be linear relationship for $pH > 1$, and a second-order rate constant of 2.0×10^{-4} M^{-1} s^{-1} can be deduced. However, the point at $pH < 1$ is considerably below the line.



hydrolysis of nocardicin A at 37°C in dilute HCl

FIGURE 22



hydrolysis of nocardicin A at 30° C
in moderately concentrated H_2SO_4

FIGURE 23

Table 42. Hydrolysis of nocardicin A in dilute HCl at 37°C.

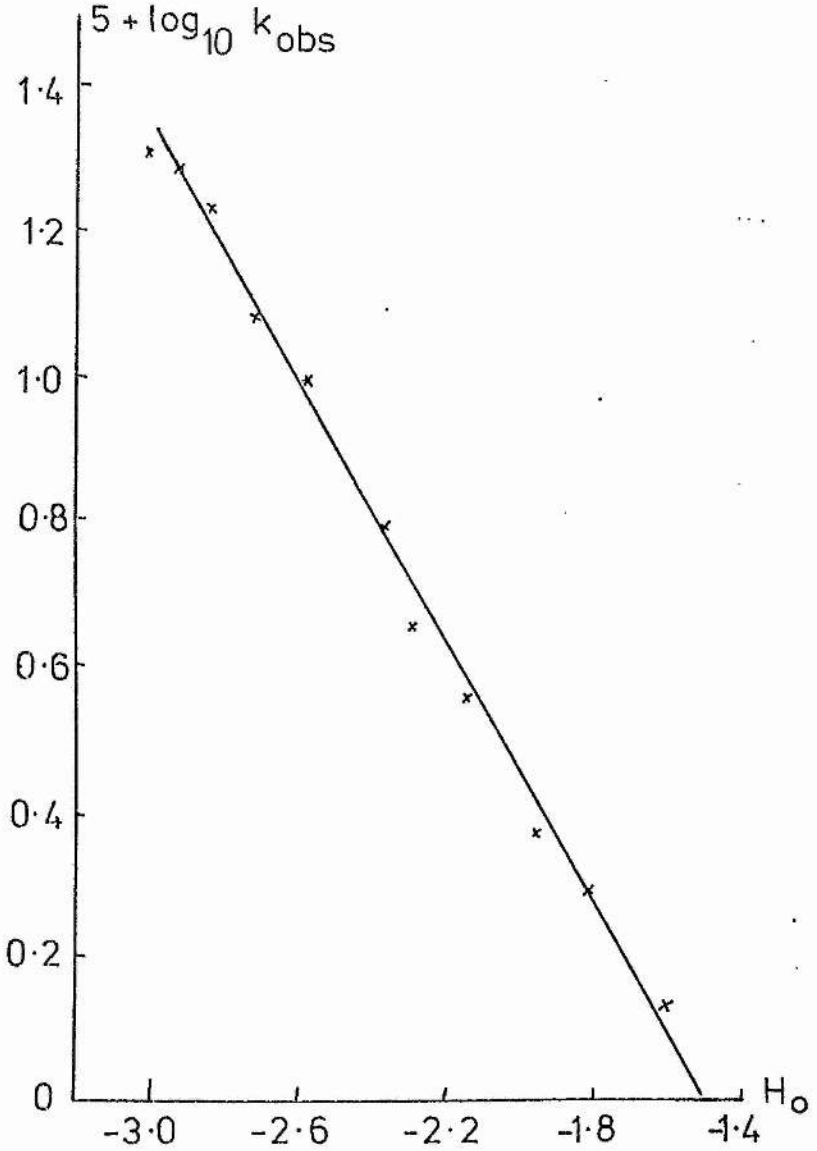
pH	H^+/M	$10^5 k_{obs}/s^{-1}$
0.76	0.173	2.85
1.05	0.089	1.80
1.15	0.07	1.40
1.31	0.048	0.90

A further set of rate constants were measured in moderately concentrated sulphuric acid solutions. The temperature in this case was 30°C. The spectral changes observed in these experiments are identical with those for the reaction in dilute acid.

Table 43. Hydrolysis of nocardicin A in moderately concentrated H_2SO_4 at 30°C.

% acid w/w	$-H_o$	$10^4 k_{obs}/s^{-1}$	$4 + \log_{10} k_{obs}$
45.5	3.00	25.5	1.40
44.0	2.86	22.5	1.35
42.5	2.73	20.5	1.31
40.5	2.58	17.0	1.23
33.5	2.08	8.35	0.92
31.0	1.89	6.35	0.80
29.0	1.75	5.50	0.74
26.0	1.54	4.00	0.60

Plotting $\log_{10} k_{obs}$ against H_o gives a straight line (figure 23) whose gradient is - 0.57.



hydrolysis of N-phenylazetidin-2-one at 30°C in mod. conc. H_2SO_4

FIGURE 24

N-PHENYLAZETIDIN-2-ONE

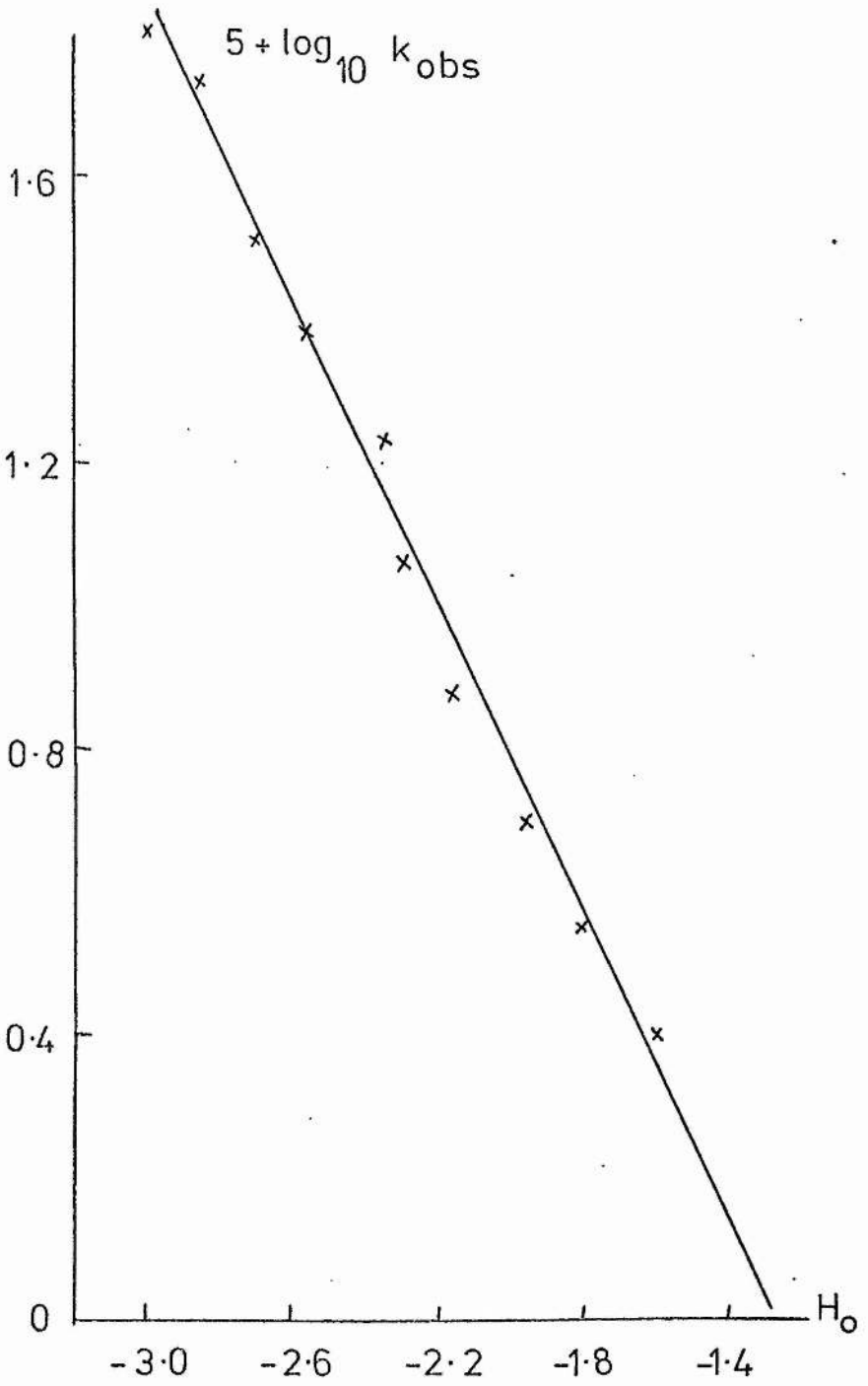
The ultraviolet spectrum of a solution of N-phenylazetidin-2-one (6.8×10^{-5} M) in 5N HCl was taken. The λ_{max} was at 246 nm, ($\varepsilon_{246} = 22{,}000$). This peak was observed to decline over a period of four hours.

The diminution of this absorption was used to obtain the psuedo-first-order rate constant for the acid-catalysed ring-opening of the compound.

Table 44. Reaction of N-phenylazetidin-2-one in moderately concentrated H_2SO_4 at 30°C.

$-H_o$	$10^5 k_{obs}/s^{-1}$	$5+ \log_{10} k_{obs}$
3.00	20.5	1.311
2.86	17.0	1.230
2.73	12.5	1.079
2.58	9.75	0.989
2.37	6.2	0.792
2.30	4.45	0.648
2.15	3.6	0.556
1.96	2.35	0.371
1.82	1.95	0.290
1.61	1.35	0.130

A plot of $\log_{10} k_{obs}$ against H_o is shown in figure 24. This is a straight line, with gradient 0.87. Since the gradient is not unity, a second-order rate constant cannot be derived. An approximate value would be 4.4×10^{-7} M^{-1} s^{-1}.



hydrolysis of
N-p-nitrophenylazetidin-2-one
at 30°C in mod.conc. H_2SO_4

FIGURE 25

p-NITROPHENYLAZETIDIN-2-ONE

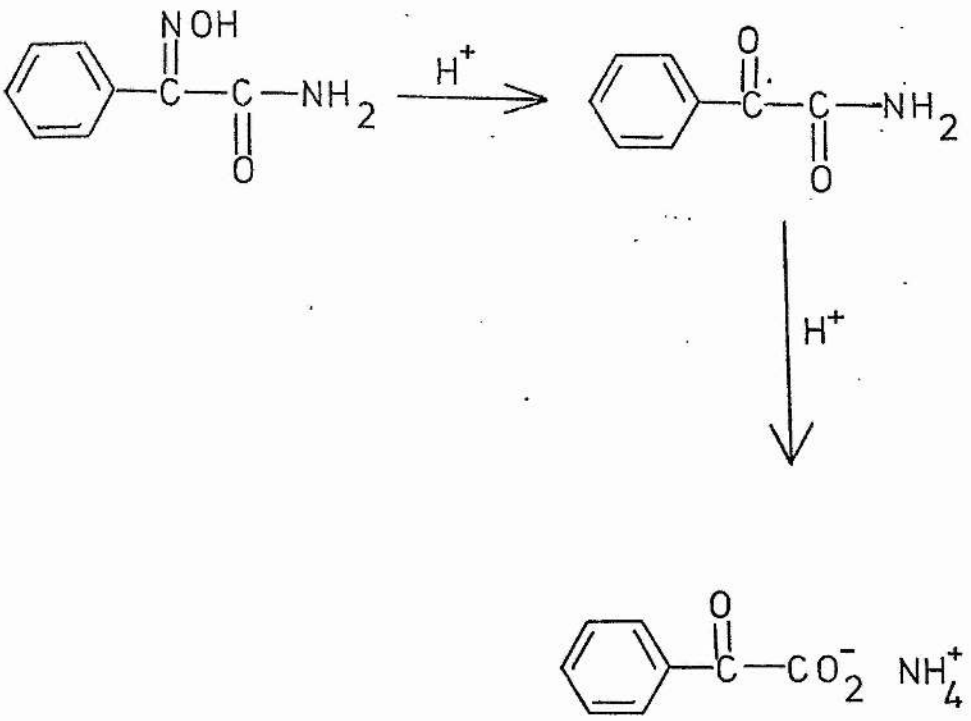
The ultraviolet spectrum of a solution of N-p-nitrophenylazetidin-2-one (1.04×10^{-4} M) in 4N HCl was taken. The λ_{max} was at 327 nm ($\varepsilon_{327} = 14{,}400$). This peak was observed to decline over a period of three hours.

The diminution of this absorption was used to obtain psuedo-first-order rate constants for the acid-catalysed ring-opening of the compound.

Table 45. Reaction of N-p-nitrophenylazetidin-2-one in moderately concentrated H_2SO_4 at 30°C.

$-H_o$	$10^5 k_{obs}/s^{-1}$	$5 + \log_{10} k_{obs}$
3.00	63.0	1.799
2.86	54.5	1.736
2.73	32.5	1.512
2.58	24.0	1.380
2.37	17.0	1.230
2.30	11.5	1.061
2.15	7.5	0.875
1.96	5.0	0.699
1.82	3.55	0.550
1.61	2.5	0.398

A plot of $\log_{10} k_{obs}$ against H_o is shown in figure 25. This is a straight line, with gradient 1.07. This is close enough to 1 for a linear relationship between rate of reaction and activity of hydrogen ion to be inferred. The second-rate constant, then, is 4.6×10^{-7} M^{-1} s^{-1}.



SCHEME 31

BENZIL MONO-OXIME

The ultraviolet spectrum of benzil mono-oxime has its maximum at 257 nm. In a sulphuric acid solution of H_o = -3.00, this maximum was observed to shift slightly to 263 nm over a period of four hours. It was felt that this spectral change was too slight to warrant further kinetic studies.

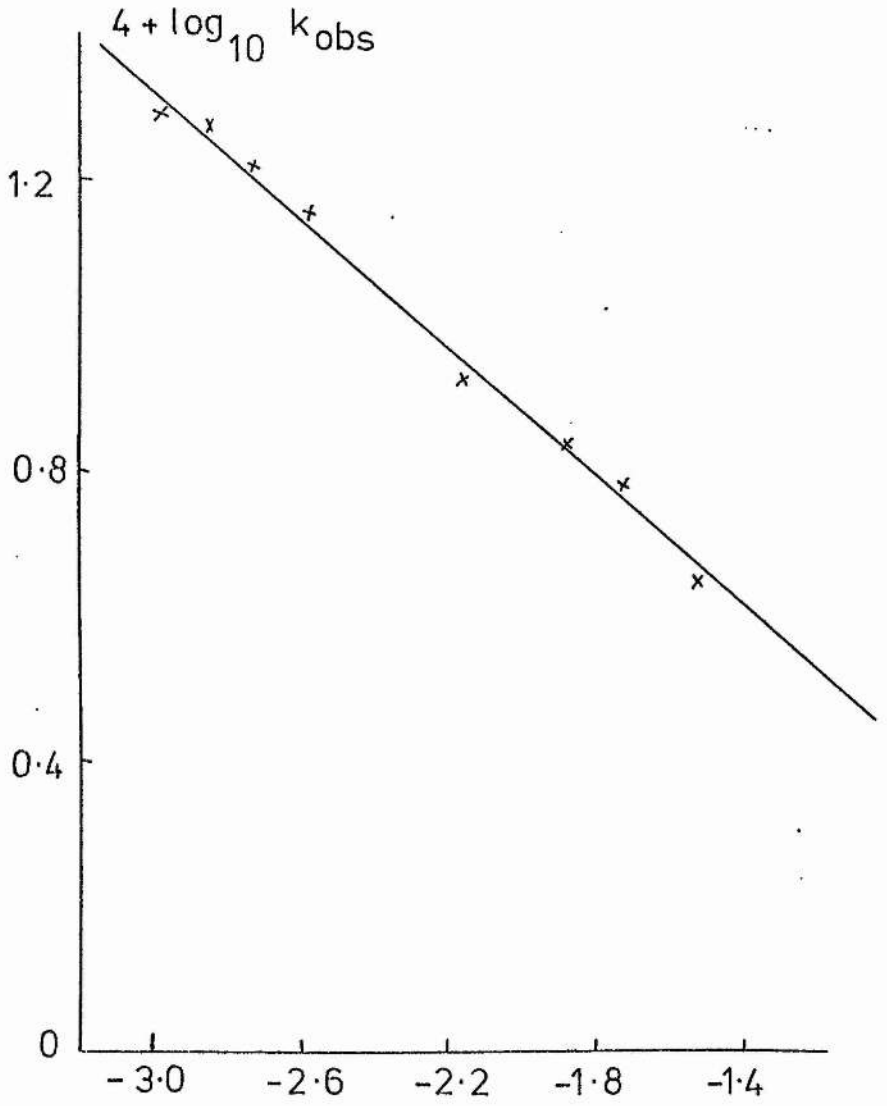
BENZOYL FORMAMIDE MONO-OXIME

The ultra violet spectrum of benzoyl formamide mono-oxime has its maximum absorption at 250 nm. In a sulphuric acid solution of H_o = -3.00 this maximum shifts to 260 nm. However, the spectral change exhibits no isosbestic points. It is assumed that this shift corresponds to hydrolysis of the oxime function and consequent formation of benzoyl formamide. An authentic sample of benzoyl formamide was indeed found to have its ultra-violet maximum at 260 nm in an acidic solution (250 nm in neutral solution) However, over a period of one day, the intensity of this peak markedly increased. (Extinction coefficient increased from 5,400 to 9,700.) It is assumed that this change corresponds to hydrolysis of the amide to benzoyl formic acid. And, indeed, an authentic sample of benzoyl formic acid was found to have its ultraviolet maximum at 260 nm, with ε_{260} = 9,200. Thus the evidence is consistent with the changes summarised in scheme 31.

The diminution of absorption at 250 nm was used to obtain psuedo-first-order rate constants for the hydrolysis of the oxime. The results are in table 46.

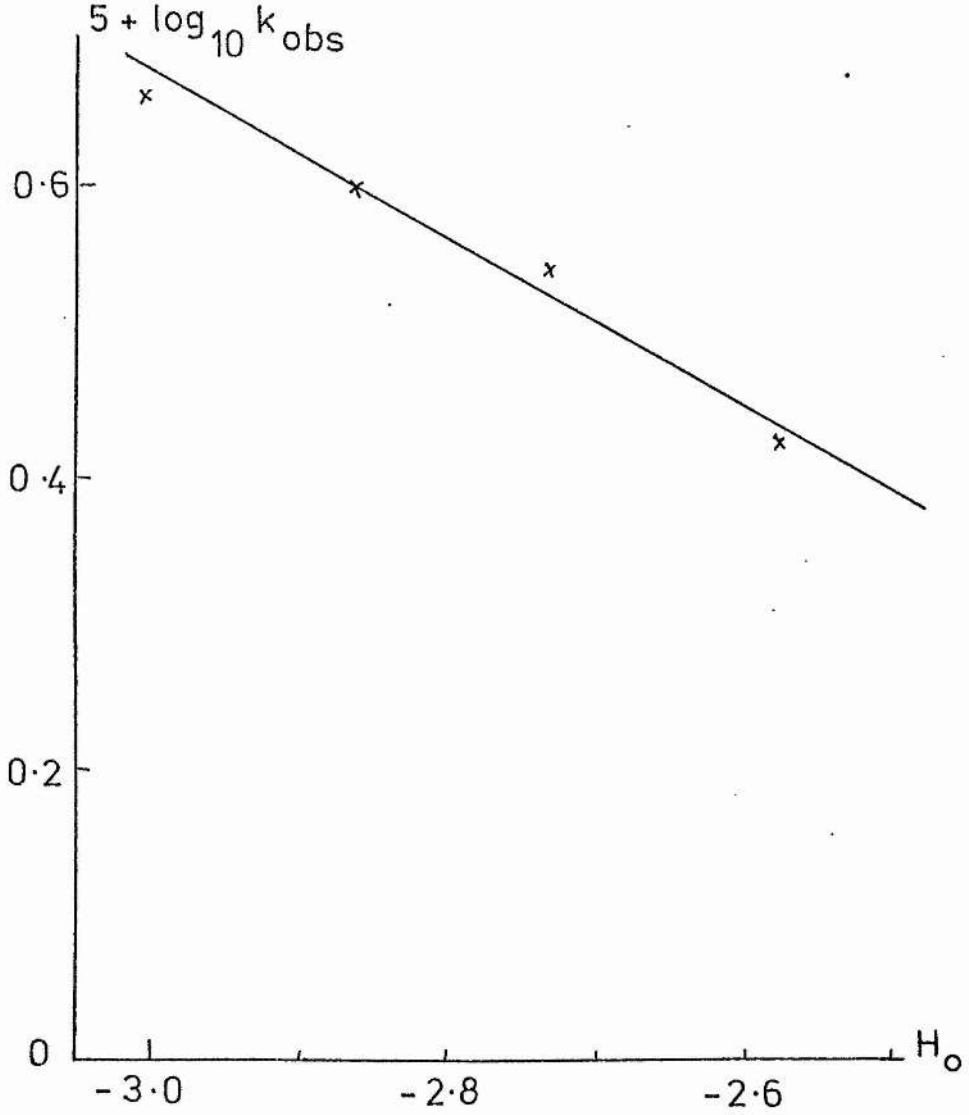
A plot of $\log_{10} k_{obs}$ against H_o is shown in figure 26. This is a straight line with gradient - 0.47.

The increase in absorption at 260 nm in the spectrum of benzoyl formamide was used to obtain psuedo-first-order rate constants for the



hydrolysis of benzoyl formamide oxime at 30°C in mod. conc. H_2SO_4

FIGURE 26



hydrolysis of benzoyl formamide at 30°C in mod. conc. H_2SO_4

FIGURE 27

amide hydrolysis. The results are in table 47.

Table 46. Hydrolysis of benzoyl formamide mono-oxime in moderately concentrated H_2SO_4 at 30°C.

$-H_o$	$10^4 k_{obs}/s^{-1}$	$4 + \log_{10} k_{obs}$
3.00	20.0	1.30
2.86	19.0	1.28
2.73	16.5	1.22
2.58	14.5	1.16
2.08	8.45	0.93
1.89	6.90	0.84
1.75	6.00	0.78
1.54	4.50	0.65

Table 47. Hydrolysis of benzoyl formamide in moderately concentrated H_2SO_4 at 30°C.

$-H_o$	$10^5 k_{obs}/s^{-1}$	$5 + \log_{10} k_{obs}$
3.00	4.6	0.633
2.86	4.0	0.602
2.73	3.5	0.544
2.58	2.65	0.423

A plot of $\log_{10} k_{obs}$ against H_o is shown in figure 27. This is a straight line with gradient -0.65.

The rates of amide hydrolysis are roughly forty times slower than those of oxime hydrolysis; therefore the effect of the former process on the kinetics of the latter will be negligibly small.

DISCUSSION

The acid-catalysed reaction of nocardicin A is known to consist of two processes: the oxime hydrolysis and the β-lactam hydrolysis. These, it may be supposed, take place quite independently of each other. It is therefore a little surprising that the spectral changes observed during the transformation are so straightforward. In particular, the existence of tight isosbestic points would suggest that one single reaction was taking place. The explanation most probably is that only one step is detectable by changes in the UV spectrum. This is most probably the oxime hydrolysis, as β-lactam hydrolysis occasions no significant spectral changes. (This is amply demonstrated by the observation that the spectrum of nocardicin A does not change on standing in alkali. The alkali cleaves the β-lactam ring, but does not affect the oxime function.)

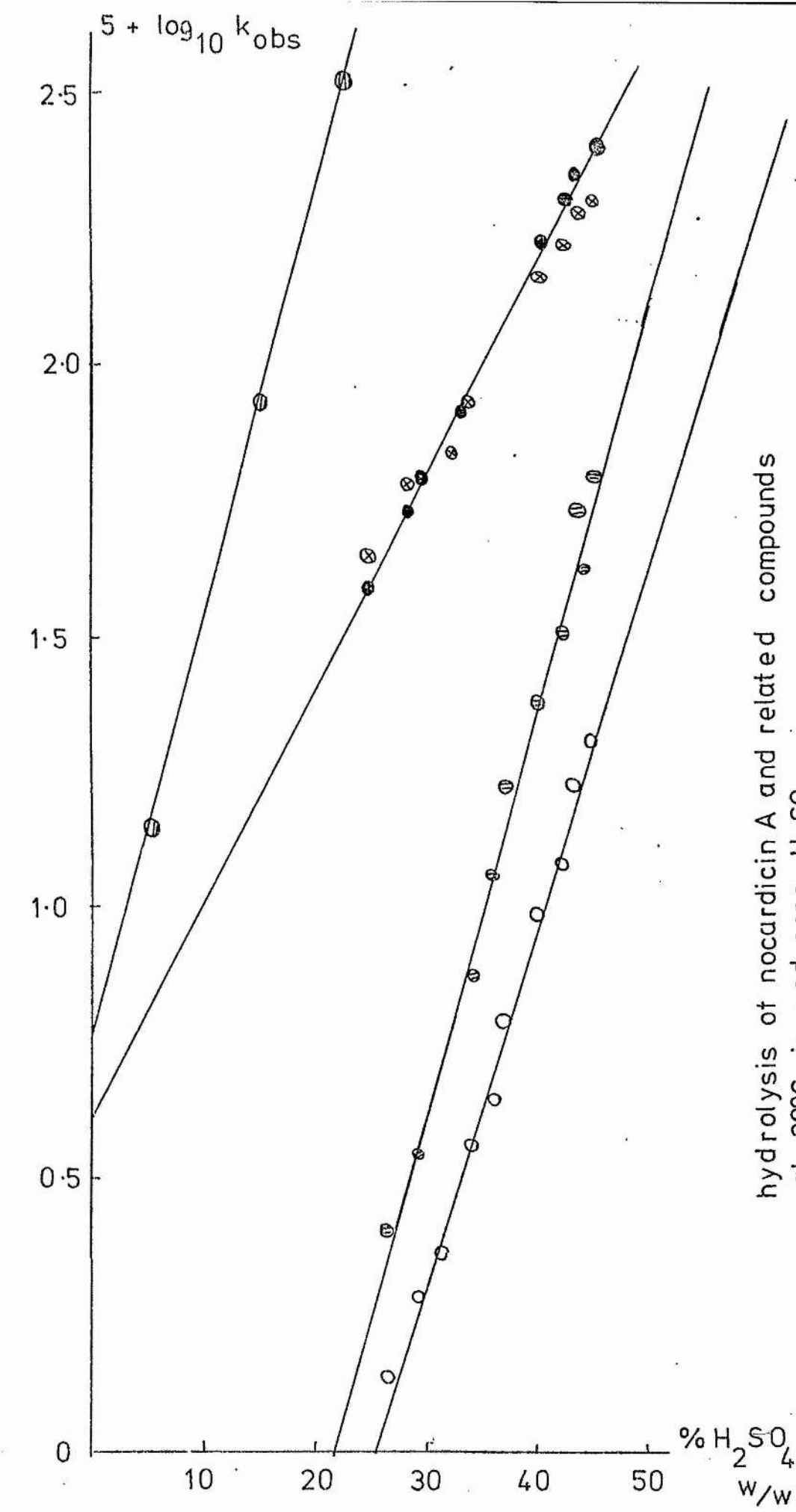
The results do indicate that the reaction of this antibiotic is remarkably slow; its acid stability is therefore far superior to that enjoyed by any penicillin. Compare, for example, the behaviour of benzylpenicillin under similar conditions. At pH 1.32 and 30°C it degrades with a rate constant of 4.5×10^{-3} s^{-1}. This is four hundred times faster than the reaction of nocardicin A, even at the lower temperature. Penicillin V, at pH 1.26 and 30°C has a rate constant of 1.5×10^{-4} s^{-1}. This acid-stable penicillin reacts thirteen times faster than nocardicin A.

The evidence overwhelmingly suggests that these rate constants do refer specifically to the oxime hydrolysis, and tell us little about the β-lactam hydrolysis. This is bourne out by the following observations.

1. The spectral changes observed for the nocardicin reaction are qualitatively the same as those for the reaction of benzoyl formamide mono-oxime (52). Only the position of the peaks is different. The changes for the benzil mono-oxime reaction are also similar. In this case they are very much smaller, probably because the spectrum is dominated by the two conjugated benzene rin which dwarf any differences between the ketone and its oxime.

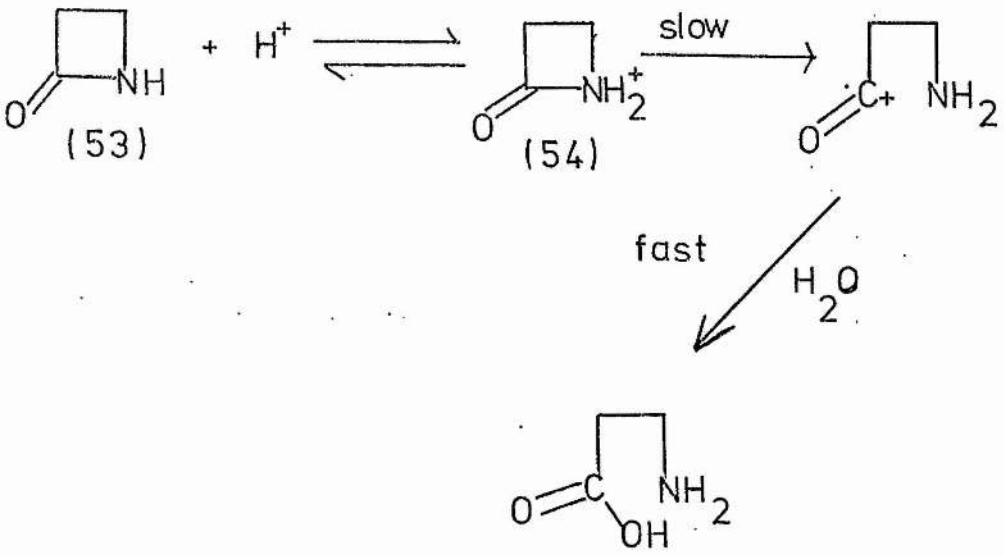
2. The rates of reaction of nocardicin A in moderately concentrated sulphuric acid are of the same order of magnitude as those of benzoyl formamide mono-oxime (52). They are less than a factor of two higher. There are no structural features of nocardicin A which would lead one to expect its oxime function to hydrolyse at a rate greatly different from that of (52).

Comparison of values of the rate constants for nocardicin A and the model β-lactams are complicated because the relationship changes with acidity. At H_o = -3.00, N-p-nitrophenylazetidin-2-one (50) reacts four times slower than nocardicin. However, it is to b expected that nocardicin's β-lactam ring will hydrolyse much more rapidly than (49) or (50). In the latter compounds the aromatic system will considerably reduce the basicity of the nitrogen, making these compounds less reactive towards acid than azetidin-2-one itself. The N-substituent in nocardicin would not have this effect. Data on the reaction of azetidin-2-one (53) with acid is available from the work of Yates et al.[84], some of which is tabulated in table 48.



hydrolysis of nocardicin A and related compounds at 30°C in mod. conc. H_2SO_4
⊘-(8); ●-(1); ⊗-(7); ⊖-(5); O-(4)

FIGURE 28



SCHEME 32

Table 48. Hydrolysis of azetidin-2-one at 33°C in aqueous H_2SO_4.

% acid w/w	5.1	15.5	22.5	32.7	41.8
$10^4 k_{obs}/s^{-1}$	1.39	8.62	34.4	88.5	272

These results show that, in the acidity range in which the nocardicin reaction was studied, (53) reacts between 9 and 16 times faster than nocardicin. There is no reason to believe that the β-lactam in nocardicin will react slower than (53); if anything, one would expect it to react faster still. This is because there is a possibility of assistance to the ring-cleavage process from the neighbouring amide carbonyl. (cf the participation of the side-chain carbonyl in ring-cleavage reactions of penicillin.)

3. The variation of rate with acidity for nocardicin is similar to that obtained for benzoyl formamide oxime (52): i.e. a large increase in acidity produces only a slight increase in rate. In contrast, the model β-lactams (49), (50) and (53), are much more sensitive to change in acidity. This is illustrated in figure 28.

In summary, the spectral changes observed during the reaction of nocardicin A with acid are exactly what would be expected for a compound wit that type of oxime structure, regardless of whether or not it contained a β-lactam ring.

Finally, some remarks may be made concerning the mechanism of these processes. Yates et al.[84] produce good evidence that β-lactams, in contra to other lactams, react by the mechanism shown in scheme 32. Although protonation occurs predominantly on oxygen[108], there is some N-protonation[10] and the slow step is the ring-opening of this species (54). Thus, (53) bein

an extremely weak base, the rate is proportional to the activity of hydrogen ion throughout the acidity range. Lactams of larger size exhibit a maximum in their rate profiles, indicating a mechanism in which the activity of water plays an important role. But it seems reasonable to assume that hydrolysis of the β-lactam ring of nocardicin A occurs by the same mechanism as for the other β-lactams.

Nocardicin A exhibits similar behaviour to that of benzoyl formamide oxime; however, this behaviour is most certainly not typical of oximes generally.

The hydrolysis of acetophenone oxime (55) in acid has been considered in detail by Gregory and Moodie[110]. They find that the rate decreases with increasing concentration of sulphuric acid, and is reasonably constant between pH 1 and H_o -0.43. Some of their data is in tables 49 and 50.

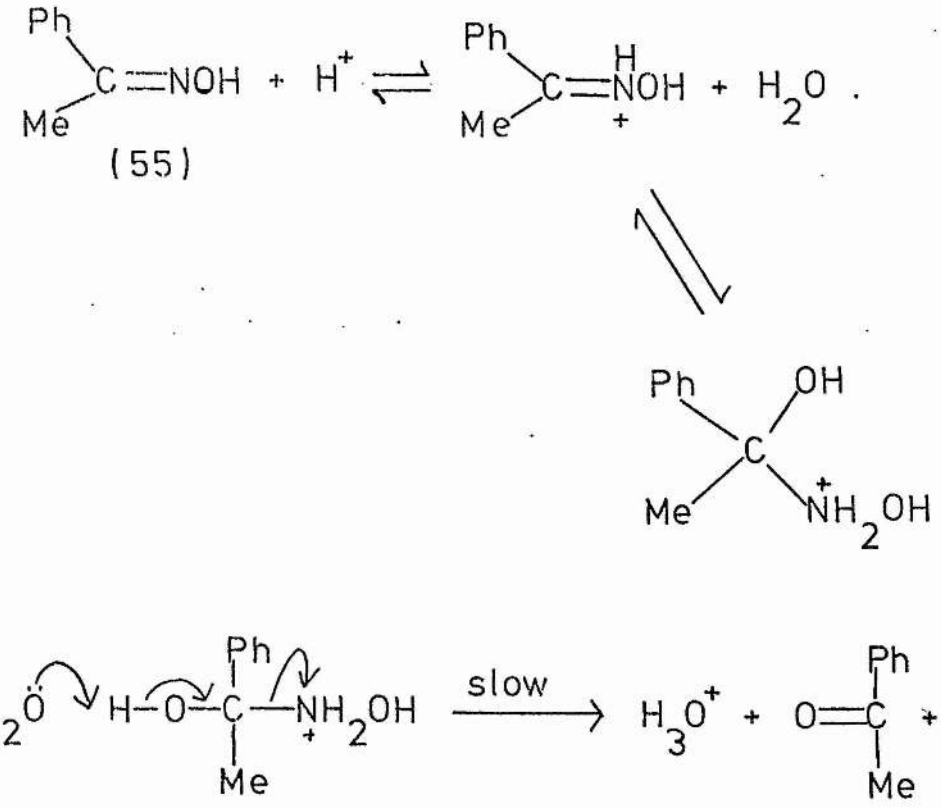
Table 49. Hydrolysis of acetophenone oxime at 25°C in aqueous H_2SO_4.

% acid w/w	60.6	55.0	49.7	39.8	29.5	22.9	10.
$10^4 k_{obs}/s^{-1}$	0.064	0.16	0.290	0.69	1.30	1.81	3.

Table 50. Hydrolysis of acetophenone oxime at 25°C in aqueous HCl.

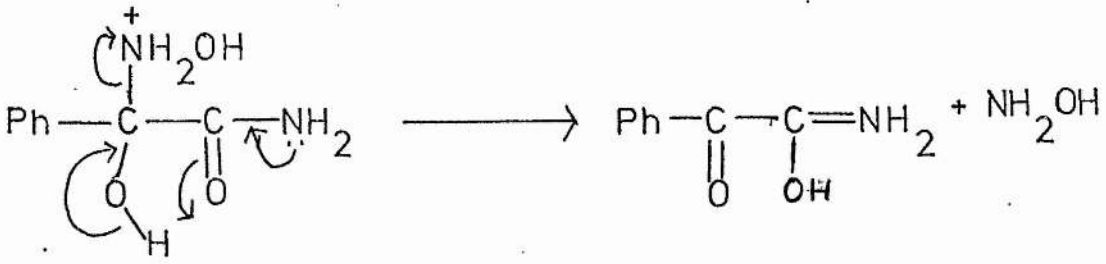
$[H^+]/M$	0.47	0.235	0.094	0.0376
$10^4 k_{obs}/s^{-1}$	4.9	5.0	4.7	2.95

To account for this, the mechanism of scheme 33 is proposed. There is independent evidence that protonation of an oxime occurs on the nitrogen[111]. Because two steps (including the slow one) involve water, the rate of reaction in moderately concentrated acid is affected, not only by the acidity, but also by the activity of water, which falls with

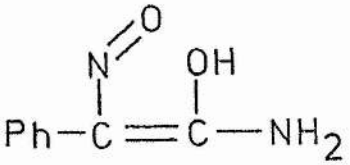


(55)

SCHEME 33



SCHEME 34



(56)

increasing acid concentration. The pKa of oximes appears to be about 3[112, 113], therefore throughout the range considered the protonation step is essentially complete. In dilute acid the activity of water is invariant, therefore the rate is also constant. Similar results have been obtained for the hydrolysis of cyclohexanone and cyclopentanone oximes[113, 114].

The rate of hydrolysis of nocardicin and benzoyl formamide oxime are considerably higher than the maximum found for acetophenone oxime, and the rate-acidity profile is completely different. This betokens a different mechanism of oxime hydrolysis, doubtless resulting from the proximity of the amide function.

The observed increase in rate with acidity throughout the range indicates that the pKa of these oximes are considerably lower than usual, so that the concentration of protonated oxime increases with acidity. In the pH range, this gives rise to the linear dependence of rate on hydrogen ion activity. In the H_o range the extra factor of a decreasing activity of water makes the rate less sensitive to changes in acidity.

One reason for the reduced basicity of these oximes may be that hydrogen bonding in (52) between the hydroxyl group and the carbonyl of the amide leads to a structure with (56) as one of its canonical forms. The nitrogen of the N = O group is only weakly basic and so it is not unreasonable to propose that (52) is a weaker base than (55).

Although the presence of an amide group lowers the basicity of (52), it still hydrolysis more rapidly than (55). It could be that the slow step in oxime hydrolysis, proton removal from the tetrahedral intermediate, is effected in this case not by a water molecule (as in scheme 33) but by the neighbouring amide group (scheme 34). Thus the amide group in benzoylformamide mono-oxime and nocardicin A may exercise two functions:

reducing the basicity of the oxime, but enhancing the reactivity of the hydrated form.

What is significant in our understanding of the overall acid hydrolysis of nocardicin A is that observed specral changes are due to oxime hydrolysis, not to opening of the β-lactam ring. From the foregoing, it may be concluded that, in moderately concentrated acid, the mechanism of nocardicin A hydrolysis is relatively rapid opening of the β-lactam ring and slower hydrolysis of the oxime group. As these two processes vary with acidity in different ways it may be that, at lower acidities, β-lactam cleavage is the slower process. Neither process occurs at high pH, such as that in biological systems, and so the acid hydrolysis of nocardicin A is not important in its use as an antibiotic.

APPENDICES

APPENDIX 1

KEDZY - SWINBOURNE METHOD FOR THE DETERMINATION OF FIRST-ORDER RATE CONSTANTS[76].

Any first-order reaction must obey equation (i),

$$A = A_o e^{-kt} \qquad - (i)$$

where A is the concentration of the reacting species at time t, A_o is its initial concentration and k is the first-order rate constant. At a later time $(t + \Delta t)$, the concentration of reactant would be A^1, where

$$A^1 = A_o e^{-k(t + \Delta t)} \qquad - (ii)$$

Dividing (i) by (ii) gives (iii).

$$A/A^1 = e^{k \Delta t} \qquad - (iii)$$

or

$$A = A^1 e^{k \Delta t} \qquad - (iv)$$

Thus if the concentration of the reactant is known at two points a known time apart, equation (iv) allows k to be calculated. The usual procedure is to take a number of pairs of concentrations (A and A^1), separated by a fixed time interval Δt. A graph is plotted of A as a function of A^1, the gradient (g) is measured, and then

$$k = (\ln g)/\Delta t \qquad - (V)$$

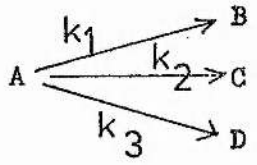
t is chosen to be ca. 70% of the half-life.

It is not necessary to use the absolute values of A in this analysis: any set of measurements which have a linear relationship with the concentrations will yield the same rate constant. The displacement from the base-line on graphs of optical density against time was used in this project.

APPENDIX 2

A MATHEMATICAL MODEL OF SIMULTANEOUS REACTIONS.

Assume that a substance A reacts in a number of different ways simultaneously. The case of 3 different pathways is considered here, but the conclusions can be generalised to any number.



The concentrations of these substances can be formulated.

$$[A] = [A]_o e^{-k_T t} \quad \text{- (i)}$$

where $k_T = k_1 + k_2 + k_3$ - (ii)

$$\frac{d[B]}{dt} = k_1[A] \quad \text{- (iii)}$$

$$\Rightarrow d[B]/dt = k_1[A]_o e^{-k_T t} \quad \text{- (iv)}$$

$$\Rightarrow [B] = \frac{k_1}{k_T}[A]_o (1 - e^{-k_T t}) \quad \text{- (v)}$$

Similarly,

$$[C] = \frac{k_2}{k_T}[A]_o (1 - e^{-k_T t}) \quad \text{- (vi)}$$

$$[D] = \frac{k_3}{k_T}[A]_o (1 - e^{-k_T t}) \quad \text{- (vii)}$$

Now,

$$\frac{k_1}{k_T}[A]_o = [B]_\infty \quad \text{- (viii)}$$

Therefore,

$$\ln([B]_\infty - [B]) = -k_T t \quad \text{- (ix)}$$

Thus, plotting a graph of $\ln([B]_\infty - [B])$ vs t allows k_T, and not k_1, to be found. Consideration of the concentrations of the other products will also yield k_T.

A Swinbourne analysis of data relating to $[B]$ (or $[C]$ or $[D]$) will also yield k_T, since, for sets of measurements $[B]$ and $[B]'$ separated by a fixed time interval Δt,

$$[B] = [B]' e^{k_T \Delta t} + [B]_\infty (1 - e^{-k_T \Delta t}) \qquad - (x)$$

Therefore, it makes no difference whether a reactant or product is observed during a kinetic experiment: the analysis always yields the rate constant for the consumption of the reactant.

APPENDIX 3

A MATHEMATICAL MODEL FOR TWO CONSECUTIVE REACTIONS.

For the generalised reaction scheme

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

where k_1 and k_2 are the rate constants for the reactions of A and B respectively, the concentrations of the three substances obey the following laws.

$$[A] = [A]_o \, e^{-k_1 t} \qquad \text{- (i)}$$

$$[B] = \frac{k_1}{k_2 - k_1} [A]_o \, (e^{-k_1 t} - e^{-k_2 t}) \qquad \text{- (ii)}$$

$$[C] = [A]_o + \frac{1}{k_2 - k_1} [A]_o \, (k_1 e^{-k_2 t} - k_2 e^{-k_1 t}) \qquad \text{-(iii)}$$

In this project, much of the acquired data has been for the concentration of an intermediate. Therefore the most relevant equation is (ii). This can be simplified as follows

Assume $k_1 \gg k_2$.

Then, for sufficiently small values of t,

$$e^{-k_2 t} \approx 1.$$

Therefore, $[B] = [A]_o \, (1 - e^{-k_1 t})$. - (iv)

Thus, an analysis of data acquired close to the beginning of the reaction yields k_1.

Also, for sufficiently large values of t,

$$e^{-k_1 t} \approx 0$$

Therefore,

$$[B] = [A]_o e^{-k_2 t}. \qquad -(v)$$

Thus, an analysis of data acquired towards the end of the reaction yields k_2.

Now suppose that $k_1 \ll k_2$.

Then, for sufficiently small values of t,

$$e^{-k_1 t} \approx 1.$$

Therefore,

$$[B] = \frac{k_1}{k_2} A_o (1 - e^{-k_2 t}). \qquad - (vi)$$

An analysis of data acquired close to the beginning of the reaction will therefore yield k_2.

And, for sufficiently large values of t,

$$e^{-k_2 t} \approx 0$$

Therefore,

$$[B] = \frac{k_1}{k_2} A_o e^{-k_1 t} \qquad - (vii)$$

An analysis of data acquired close to the end of the reaction will therefore yield k_1.

In summary, providing the two rate constants are sufficiently different, they can both be elucidated from a graph of [B] *vs* time. For this approximate method to be useful, k_1 and k_2 must be different by at least a factor of 4. Even so, though, the higher of the two will always

appear, from this analysis, to be ca. 20% higher than it actually is.

The rising part of the graph always yields the higher rate constant, and the falling part the lower one, regardless of which process occurs first

The time taken for [B] to reach a maximum can be found by differentiating equation (ii) and setting the expression equal to zero.

$$\frac{d[B]}{dt} = \frac{k_1}{k_2 - k_1} [A]_o (k_2 e^{-k_2 t} - k_1 e^{-k_1 t}) = 0 \qquad \text{- (viii).}$$

$$\Longrightarrow \qquad t_{max} = \frac{1}{k_2 - k_1} \ln \frac{k_2}{k_1} \qquad \text{- (ix).}$$

APPENDIX 4

A FORTRAN COMPUTER PROGRAM FOR THE ANALYSIS OF DATA FOR TWO CONSECUTIVE REACTIONS.

The optical density at any time is given by

$$O.D. = \varepsilon_A[A] + \varepsilon_B[B] + \varepsilon_C[C]$$

where ε isthe extinction coefficient of the substance. The concentrations of each species are found from equations (i) - (iii) of Appendix 3.

The text of the program is as follows:

```
      IMPLICIT REAL *8(A-H,O-Z)
      DIMENSION BL(3),BU(3),W(40),X(3)
      INTEGER IW(6)
      COMMON/A/NPTS
      COMMON/A/ITIME(100)
      COMMON/A/DT(100)
      COMMON/A/EC
      N=3
      READ(1,10)NPTS
   10 FORMAT(I6)
      READ(1,12)(X(K),K=1,3)
   12 FORMAT(3D10.3)
      READ(1,12)(BL(K),K=1,3)
      READ(1,12)(BU(K),K=1,3)
      READ(1,13)EC
```

```
 13 FORMAT(D10.3)
    READ(1,11)(ITIME(K),K=1,NPTS),(DT(K),K=1,NPTS)
 11 FORMAT( NPTS I6, NPTS F6.3)
    IBOUND=0
    LIW=5
    LW=40
    IFAIL=1
    CALL E04JAF(N,IBOUND,BL,BU,X,F,IW,LIW,W,LW,IFAIL)
    IF(IFAIL.NE.0)WRITE(6,97)IFAIL
 97 FORMAT('IFAIL= ',I3)
    IF(IFAIL.EQ.1)GO TO 20
    WRITE(6,99)FC
 99 FORMAT('0',D10.3)
    WRITE(6,100)(X(J),J=1,N)
100 FORMAT('0',D10.3,2X,D10.3,2X,D10.3)
    STOP
    END
    SUBROUTINE FUNCT1(N,XC,FC)
    IMPLICIT REAL *8(A-H,O-Z)
    DIMENSION XC( 3 )
    COMMON/A/NPTS
    COMMON/A/ITIME(100)
    COMMON/A/DT(100)
    COMMON/A/EC
    X1=XC(1)
    X2=XC(2)
    X3=XC(3)
```

```
      DO=DT(1)
      FC=0.0
      DO 1 I=2,NPTS
      T=DFLOAT(ITIME(I))
      D=DT(I)
      FT=(D-DO)/X1-X2*(DEXP(-X2*T)-DEXP(-X3*T))/(X3-X2)
      FD=EC (1+(X2 DEXP(-X3*T)-X3 DEXP(-X2*T))/(X3-X2))/X1
      FT=FT-FD
    1 FC=FC+FT*FT
      RETURN
      END
```

This program uses a least-squares minimisation process to find the optimum values of three parameters: X(1), X(2) and X(3). X(2) and X(3) are the two rate constants k_1 and k_2. X(1) is the product of ε_B, $[A]_o$ and the fraction of A which is transformed into B.

The data required for the program is, in order:

1. The number of data points (NPTS) to be used
2. Initial guesses for X(1), X(2) and X(3).
3. Lower bounds set on the values of these parameters (BL(1), BL(2) and BL(3))
4. Upper bounds set on the parameters (BU(1), BU(2), BU(3))
5. An estimate of the final absorbance of the solution (EC). (This is usually extremely small, and is $O.D._\infty - O.D._o$.)
6. The experimental data. This consists of a list of all the times to be considered, followed by a list of all the optical densities measured at these times. There should be NPTS items in each list. All of this data constitutes one statement.

For each data point, the program evaluates FT, which is the difference between the experimental optical density and the value calculated using the current values of X(1), X(2) and X(3) and time. It then evaluates FC, which is the sum of the squares of FT over all NPTS data points. And it finds and prints out those values of X(1), X(2) and X(3), within the prescribed bounds, which give rise to the lowest FC. To do this, the program makes use of an algorithm (E04JAF), which is part of the St Andrews University computer's permanent software, on the NAG files.

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